ids. Although multiple functions have been suggested for these proteins, their true physiological roles have not yet been elucidated. They have been implicated in phospholipase A_2 regulation (*16*), membrane trafficking (*17*), cytoskeletal organization (*18*), and blood coagulation (*19*). In many cases the proposed function requires that the proteins be secreted, yet these proteins lack signal sequences and are found within cells. In addition, many of the ascribed functions can be attributed to the metal- or lipid-binding properties of these molecules, and large amounts of protein are needed. In contrast, inositol 1,2-cyclic phosphate 2-phosphohydrolase activity is catalytic with 700 mol of product formed per minute per mole of protein, suggesting that this is a physiological function. The fact that phosphohydrolase activity is affected by divalent metals has been noted previously (*7*). We now show that acidic phospholipids stimulate the enzyme.

There are numerous enzymes (more than 15) that have been shown to metabolize the water-soluble inositol phosphates produced during PI turnover. It is possible that other lipocortins are also enzymes that change the concentrations of messenger molecules during cellular activation.

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20. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Effect of Phospholipase C–γ Overexpression on PDGF-Induced Second Messengers and Mitogenesis

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Platelet-derived growth factor (PDGF) stimulates phospholipase C (PLC) activity and the phosphorylation of the γ isozyme of PLC (PLC-γ) in vitro and in living cells. The role of PLC-γ in the phosphoinositide signaling pathway was addressed by examining the effect of overexpression of PLC-γ on cellular responses to PDGF. Overexpression of PLC-γ correlated with PDGF-induced tyrosine phosphorylation of PLC-γ and with PDGF-induced breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2). However, neither bradykinin- nor lysophosphatidic acid–induced phosphoinositide metabolism was enhanced in the transfected cells, suggesting that the G protein–coupled phosphoinositide responses to these ligands are mediated by other PLC isozymes. The enhanced PDGF-induced generation of inositol trisphosphate (IP_3) did not enhance intracellular calcium signaling or influence PDGF-induced DNA synthesis. Thus, enzymes other than PLC-γ may limit PDGF-induced calcium signaling and DNA synthesis. Alternatively, PDGF-induced calcium signaling and DNA synthesis may use biochemical pathways other than phosphoinositide metabolism for signal transduction.

THE BREAKDOWN OF PIP_2 BY PLC generates at least two intracellular messengers: inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$], which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C (*1, 2*). Growth factors such as PDGF and epidermal growth factor (EGF) activate PLC, and this activation requires the intrinsic tyrosine kinase activity of the growth factor receptor (*3*). These growth factors stimulate the phosphorylation of one of the PLC isozymes, PLC-γ (*4–6*). This most likely results from a direct interaction of the growth factor receptors with PLC-γ, as purified EGF or PDGF receptors can directly phosphorylate PLC-γ on tyrosine residues (*6, 7*) and PLC-γ is associated with these receptors in growth factor–stimulated cells (*6, 8*). However, it is not clear whether PLC-γ is actually involved in the growth factor stimulation of PLC activity or whether phosphoinositide metabolism has a primary signaling role in mitogenesis.

To examine this issue, we overexpressed PLC-γ in cells by cotransfecting a mammalian expression vector containing bovine PLC-γ cDNA together with pSVneo into NIH 3T3 cells and selected clones by their resistance to geneticin (G418) (*9*). Immunoblotting with antibodies to PLC-γ (anti–PLC-γ) after PDGF treatment showed in-

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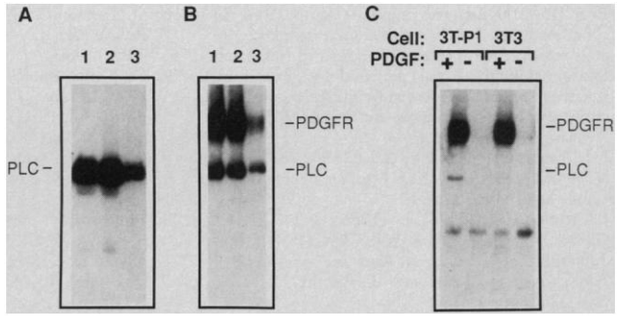

Fig. 1. Expression and PDGF-induced tyrosine phosphorylation of PLC-γ. Control 3T3 cells (pSVneo transfected only) (lanes 3) or 3T-P1 (lanes 2) and 3T-P2 (lanes 1) (PLC-γ–overexpressing cells) were grown to confluence in 15-cm dishes and treated with porcine platelet PDGF (50 ng/ml) for 2 min. Cells were lysed and subjected to immunoblotting (*10*). In both (A) and (B), 6×10^6 cells were immunoprecipitated with anti–PLC-γ antibodies. (**A**) Expression of PLC-γ. The blotting antibody was anti–PLC-γ. (**B**) PDGF-induced tyrosine phosphorylation of PLC-γ. The blotting antibody was anti-phosphotyrosine. The positions of PLC (A) and phosphorylated PLC and the PDGF receptor (PDGFR) are indicated. (**C**) Cell lysate from 8×10^5 cells was added directly to sample buffer and after SDS-PAGE immunoblotted with anti-phosphotyrosine.

creased PLC-γ expression in two transfected cell lines, 3T-P1 and 3T-P2, in which PLC-γ was increased sevenfold and fivefold over that in control cells, respectively. The extent of tyrosine phosphorylation of PLC-γ was assayed by immunoblotting with anti–PLC-γ or antibodies to phosphotyrosine (anti-phosphotyrosine) (*10*) (Fig. 1). Concomitant with increased PLC-γ expression, the PDGF-stimulated PLC-γ tyrosine phosphorylation was increased over control values, as measured by immunoblotting with anti-phosphotyrosine. Accordingly, the coimmunoprecipitation of the PDGF receptor by anti–PLC-γ was also enhanced (*6, 11*). No tyrosine phosphorylation of PLC-γ was seen in either control or overexpressing cell lines in the absence of PDGF.

We investigated the effect of increased PLC-γ expression on PDGF-stimulated PLC activity (Fig. 2). Cells were labeled with [^{3}H]myo-inositol for 60 hours and then stimulated with PDGF (purified from porcine platelets) for 30 min at 37°C in the presence of Li^+. Inositol phosphates were extracted and separated on anion exchange columns (*12*), and the sum of all inositol phosphates (Fig. 2B) and the total IP_3 pool were measured (Fig. 2C). Cells that overexpressed PLC-γ had increased PDGF-stimulated PLC activity, with increased production of total inositol phosphates (Fig. 2, A and B) and IP_3 (Fig. 2C). The increase in PDGF-stimulated inositol phosphate generation paralleled the increase in PLC-γ expression. In contrast, the responses to two agonists, bradykinin and lysophosphatidic acid, that probably activate PLC via G proteins (*13*) were not enhanced, suggesting that these agonists activate isozymes of PLC other than PLC-γ. The overexpressed PLC-γ thus appeared to be specifically activated by PDGF.

The Ca^{2+} signaling pathway of PLC-γ–overexpressing cells was examined by loading them with the fluorescent Ca^{2+} indicator indo-1 (*14*). Cells were then stimulated with PDGF, and the rise in intracellular Ca^{2+} concentration was measured. Intracellular Ca^{2+} rose to a smaller extent in the PLC-γ–overexpressing 3T-P1 cells than in the parental cells (Fig. 3) at three different concentrations of PDGF (1 ng/ml, 10 ng/ml, and 50 ng/ml). No decrease in the Ca^{2+} signal after bradykinin treatment was observed (Fig. 3), and ionomycin-releasable Ca^{2+} stores were indistinguishable between the two cell lines. Ca^{2+} signaling in PDGF-stimulated 3T-P2 cells more closely resembled the parental 3T3 cells. However, in all cell lines Ca^{2+} signaling did not correlate with PLC-γ overexpression and inositol phosphate generation.

Because the column methodology we used measures all stereoisomers of IP_3 and not just the active moiety I(1,4,5)P_3 (*15*), a direct measurement of I(1,4,5)P_3, in the absence of Li^+, was undertaken (*16*). The I(1,4,5)P_3 content of $\sim 5 \times 10^5$ cells was measured; the basal amount was <1.9 pmol per milligram of protein in both the parental 3T3 cells and 3T-P1 cells. After stimulation for 2 min with PDGF at concentrations of 20 and 50 ng/ml, the I(1,4,5)P_3 content of 3T3 cells remained <1.9 pmol per milligram of protein at both PDGF concentrations, whereas the I(1,4,5)P_3 content of 3T-P1 cells rose to 5.9 ± 0.9 and 9.9 ± 0.65 pmol per milligram of protein, respectively (mean ± SEM; n = 2 to 3 dishes). Thus, despite having a much larger intracellular content of I(1,4,5)P_3, PLC-γ–overexpressing cells did not show increases in PDGF-induced Ca^{2+} signaling. In addition, overexpression of PLC-γ and increased PIP_2 hydrolysis had a negligible influence on the mitogenic capacity of PDGF, as measured by thymidine incorporation (*17*) (Fig. 4). This is consistent with the results of others, which suggested that growth factor–induced phosphoinositide hydrolysis can be dissociated from mitogenesis (*18*). However, the possibility cannot be excluded that a certain amount of PIP_2 hydrolysis is essential for PDGF-induced mitogenesis; for example, microinjection of antibodies to PIP_2 inhibits the mitogenic response to PDGF (*19*).

Our results show that overexpression of PLC-γ leads to enhanced inositol phosphate generation in response to PDGF but not to two other agonists, bradykinin and lysophosphatidic acid. However, the increased generation of I(1,4,5)P_3 was not associated with increased PDGF-induced Ca^{2+} signaling in PLC-γ–overexpressing cell lines. Even at nonsaturating concentrations of PDGF, Ca^{2+} signaling was not enhanced in

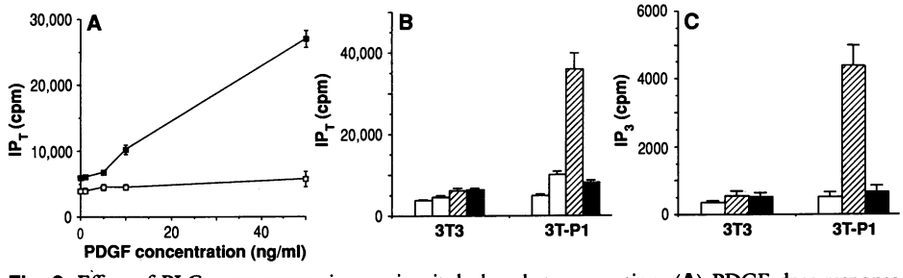

Fig. 2. Effect of PLC-γ overexpression on inositol phosphate generation. (**A**) PDGF dose-response curve. The 3T3 (open squares) and 3T-P1 (solid squares) cells were labeled with [^{3}H]myo-inositol for 72 hours (*12*). Cells were then stimulated with PDGF for 30 min in the presence of 20 m*M* LiCl and total inositol phosphates (IP_T) were measured (*12*). (Results are mean ± SEM, n = 3 or 4). (**B**) PDGF or lysophosphatidic acid–stimulated inositol phosphate generation. Control 3T3 or PLC-γ–overexpressing 3T-P1 cells were incubated with control medium (open bars), PDGF (10 ng/ml) (gray bars), PDGF (50 ng/ml) (striped bars), or lysophosphatidic acid (10 μg/ml) (solid bars) as in (A) (n = 3 to 8). At a PDGF concentration of 50 ng/ml, 3T-P2 cells gave similar results to 3T-P1. (**C**) Effect of PLC-γ overexpression on IP_3 generation in response to PDGF and bradykinin. Cells were incubated with control medium (open bars) or stimulated for 30 min at 37°C with PDGF (50 ng/ml) (striped bars) or 2 μ*M* bradykinin (solid bars) in the presence of 20 m*M* LiCl. IP_3 was measured as in (A) except an additional wash of the column with 0.4*M* ammonium formate and 0.1*M* formic acid was added (n = 4).

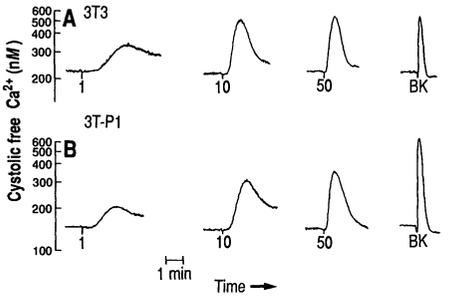

Fig. 3. Ca^{2+} signaling responses to PDGF in 3T3 and 3T-P1 cells. Representative tracings of Ca^{2+} signaling responses to PDGF at concentrations of 1, 10, and 50 ng/ml are shown in 3T3 cells (**A**) and 3T-P1 cells (**B**). Basal cytosolic Ca^{2+} was 223 ± 4.7 n*M* in the 3T3 cells and was 167 ± 3.6 n*M* (mean ± SEM, $n = 28$, in each case) in 3T-P1 cells. Peak Ca^{2+} response to PDGF (1 ng/ml) in 3T3 cells was 338 ± 12.0 n*M*, compared to 194 ± 6.4 n*M* in 3T-P1 cells ($n = 6$). Peak Ca^{2+} response to PDGF (10 ng/ml) in 3T3 cells was 462 ± 29.5 n*M*, compared to 302 ± 30.4 n*M* in 3T-P1 cells ($n = 10$). Peak Ca^{2+} response to PDGF (50 ng/ml) in 3T3 cells was 564 ± 67.3 n*M* compared to 262 ± 32.0 n*M* in 3T-P1 cells ($n = 6$). Responses to PDGF in Ca^{2+}-free medium containing 0.5 m*M* EGTA were attenuated, but similar to those shown. Assessment of total releasable Ca^{2+} stores with ionomycin in Ca^{2+}-free medium revealed no significant difference between 3T3 and 3T-P1 cells. Ca^{2+} measurements determined by fluorescence ratios in either indo-1– or fura 2–loaded adherent cells on a microscope yielded similar results. Shown for comparison is the response to 2 μ*M* bradykinin (BK) on the same cell lines.

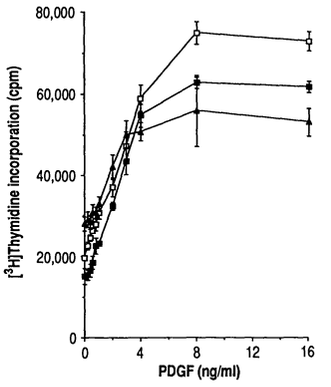

Fig. 4. PDGF stimulated thymidine incorporation in 3T3 (□), 3T-P1 (△), and 3T-P2 (■) cells. Cells were incubated for 48 hours in 0.5% FBS-containing medium and then stimulated with PDGF for 18 hours (*17*). After a 4-hour treatment with [^{3}H]thymidine, radioactivity precipitated by trichloroacetic acid was determined by scintillation counting. Results are the mean ± SEM for four dishes.

cells that overexpress PLC-γ (Fig. 3). Thus the absolute amount of IP_3 generated in response to PDGF does not correlate with the rise in intracellular Ca^{2+}. There are several possible explanations for this apparent disparity. One possibility is that I(1,4,5)P_3 production is not related to PDGF-induced Ca^{2+} signaling. Indeed, the time course of IP_3 generation is different for PDGF than for neuropeptides such as bradykinin or vasopressin, which probably signal PLC activation via G proteins (*20*). Typically, PDGF-stimulated IP_3 generation is slow and peaks in minutes, whereas neuropeptides stimulate an immediate and shorter IP_3 response. The Ca^{2+} signal in response to PDGF is also delayed compared to that in response to neuropeptides and may in fact precede the increase in I(1,4,5)P_3 (*21, 22*). Thus, the established mechanisms of classical neuropeptide Ca^{2+} signaling may not apply for PDGF signaling. As we were able to show PDGF-stimulated Ca^{2+} signaling in the presence of extracellular EGTA, at least part of the Ca^{2+} signal originates from intracellular sources.

If one assumes that I(1,4,5)P_3 is a critical event in PDGF-induced Ca^{2+} signaling, our results suggest that excess PLC-γ is compensated for by desensitization of the I(1,4,5)P_3 receptor or by increased Ca^{2+} extrusion rate. However, it is not consistent with this notion that the Ca^{2+} signal in response to bradykinin is not lower in the PLC-γ–overexpressing cells and that the ionomycin-releasable Ca^{2+} pool is not altered. Yet another possibility is that the IP_3 generated in response to PDGF is compartmentalized or sequestered within the cell and is not accessible to the Ca^{2+}-releasing organelles. Additionally, the differences observed in inositol phosphate generation between the control and PLC-γ–overexpressing cells 2 min after stimulation may not be present immediately after PDGF addition when the signal is initiated. Also, we have not examined IP_4 levels, which might have a role in Ca^{2+} signaling in these cells. The Ca^{2+} signal obtained in our study measures the average Ca^{2+} response of many cells. However, Ca^{2+} levels in single cells can be oscillatory (*1*), and the response of a cell is therefore determined by the frequency and amplitude of the oscillations. Indeed, preliminary single-cell Ca^{2+} measurements indicate that the profile of the PDGF-induced Ca^{2+} signal in individual PLC-γ–overexpressing cells is somewhat altered, but without a major change in its magnitude (*23*). The fact that we do not observe a correlation between PDGF-stimulated I(1,4,5)P_3 generation, the rise in intracellular Ca^{2+}, and mitogenesis may mean that the current view of the role of phosphoinositide metabolism as a signal for mitogenesis needs to be carefully evaluated.

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9. The expression vector pMJ-30 with the SV40 early promoter and cytomegalovirus enhancer was prepared by excising the DNA for acidic fibroblast growth factor from p267-3 [M. Jaye *et al.*, *EMBO J.* 7, 963 (1988)]. Rat brain PLC-γ DNA [P.-G. Suh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5419 (1988)] was cut from the pIBI 31 vector with Bam I and ligated to the pMJ-30 Bgl II site. NIH 3T3 2.2 cells, devoid of endogenous EGF receptors, were then transfected with PLC-γ DNA in this expression vector at a 20-fold excess over pSVneo by calcium-phosphate precipitation [M. Wigler *et al.*, *Cell* **16**, 777 (1979)]. After 48 hours, the cells were split into selection medium containing G418. Clones were selected 2 to 3 weeks later and assessed for PLC-γ expression by immunoblotting. Control 3T3 cells were transfected with pSVneo only.
10. Cells were grown to confluence on fibronectin-treated 15-cm dishes in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (10 μg/ml) at 37°C with 8% CO_2. Cells were incubated in the same medium containing 1% FBS overnight and then stimulated for 2 min with PDGF (50 ng/ml) (porcine platelet; R&D Systems) or the carrier solution of 4 m*M* HCl with bovine serum albumin (BSA) (1 mg/ml). Cells were solubilized in a 1% Triton X-100 lysis buffer containing protease and phosphatase inhibitors, and immunoprecipitated with rabbit polyclonal anti–PLC-γ (*5*). Immunoprecipitates were separated on an SDS–6% polyacrylamide gel and transferred to nitrocellulose, and immunoblotting was performed with anti–PLC-γ or anti-phosphotyrosine. Blots were incubated with ^{125}I-labeled protein A and exposed for 14 hours for autoradiography. To quantitate the degree of overexpression, bands corresponding to PLC-γ were cut from the nitrocellulose and their radioactivity content determined in a gamma counter.
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12. Cells were plated at 140,000 per well in six-well cluster plates in DMEM, 10% FBS, and penicillin and streptomycin with [^{3}H]myo-inositol (Amersham) (1 μCi/ml, 25 mCi/mmol) for 36 hours. The medium was then changed to DMEM with 1% human platelet-poor plasma and [^{3}H]myo-inositol (1 μCi/ml) for another 24 hours. Subsequently, cells were washed twice with DMEM containing 20 m*M* Hepes (*p*H 7.5), 20 m*M* LiCl, and BSA (1 mg/ml) and incubated at 37°C for 15 min. Cells were then stimulated with PDGF (50 ng/ml), 2 μ*M* bradykinin (Sigma), lysophosphatidic acid (1-oleoyl; Sigma) or PDGF carrier solution in the DMEM-Hepes-LiCl-BSA medium for 30 min at 37°C. The medium was then removed and ice-cold 5% perchloric acid was added. Cells were scraped into a small tube, and the cellular debris removed by centrifugation. The inositol phosphates were extracted from perchloric acid [E. S. Sharps and R. L. McCarl, *Anal. Chem.* **124**, 421 (1982)], then diluted into 5 ml of H_2O, and applied to Bio-Rad AG1 X8 columns [C. P. Downes, P. T. Hawkins, R. F. Irvine, *Biochem. J.* **238**, 501 (1986)]. Columns were washed with 20 ml of H_2O and 20 ml of a solution containing 5 m*M* sodium tetraborate and 60 m*M* ammonium formate, and total inositol phosphates were eluted with a solution of 1.0*M* ammonium formate and 0.1*M* formic acid. To measure IP_3, we first washed the columns with a solution of 0.4*M* ammonium formate and 0.1*M* formic acid, and then eluted IP_3 with a solution of 1.0*M* ammonium formate and 0.1*M* formic acid.
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14. Confluent monolayers of cells were suspended by brief exposure to trypsin (0.5 g/liter) and EDTA

(0.2 g/liter) in phosphate-buffered saline, followed by the addition of trypsin inhibitor and cell recovery in a nutrient medium. Indo-1-acetoxymethyl ester (2.5 μm) was then added for 30 min at 37°C, after which the cells were washed free of extracellular dye. Fluorescence measurements were performed in a Hepes-buffered physiologic saline solution at an excitation wavelength of 355 nm, with emission recorded at 400 nm, and calibration to free cytosolic Ca^{2+} concentrations as previously reported [S. Kremer *et al.*, *J. Cell Physiol.* **138**, 97 (1989); S. Kremer *et al.*, *Can. J. Physiol. Pharmacol.* 66, 43 (1988)]. Experiments were also performed on cells adherent to cover slips and loaded with fura 2 (*5*).

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16. To measure I(1,4,5)P_3, cells were grown to confluence in 60-mm dishes and then incubated for 24 hours in DMEM containing 1% human platelet-poor plasma. The medium was removed, and the cells were stimulated with PDGF (20 and 50 ng/ml) or PDGF carrier solution in DMEM containing 20 m*M* Hepes, and BSA (1 mg/ml) for 2 min at 37°C. Inositol phosphates were then extracted into 1 ml of 5% perchloric acid and neutralized (*12*). A fraction of the sample (100 μl) was assayed by a I(1,4,5)P_3 radioreceptor assay kit (Amersham).
17. Thymidine incorporation was measured by plating cells at 30,000 per well of a 24-well cluster plate in DMEM containing 10% FBS, penicillin (100μ/ml), and streptomycin (100 μg/ml) at 37°C with 8% CO_2. After 24 hours, cells were switched to the same medium containing 0.5% FBS, and after another 2 days, PDGF or carrier solution was added to the cells. After 18 hours of growth factor treatment, [^{3}H]thymidine (Du Pont, Biotechnology Systems; 0.5 μCi/ml; 8.0 Ci/mmol) was added for 4 hours. Subsequently, cells were washed and incubated with 10% trichloroacetic acid for 10 min on ice, and then the precipitated radioactivity was measured by scintillation counting.
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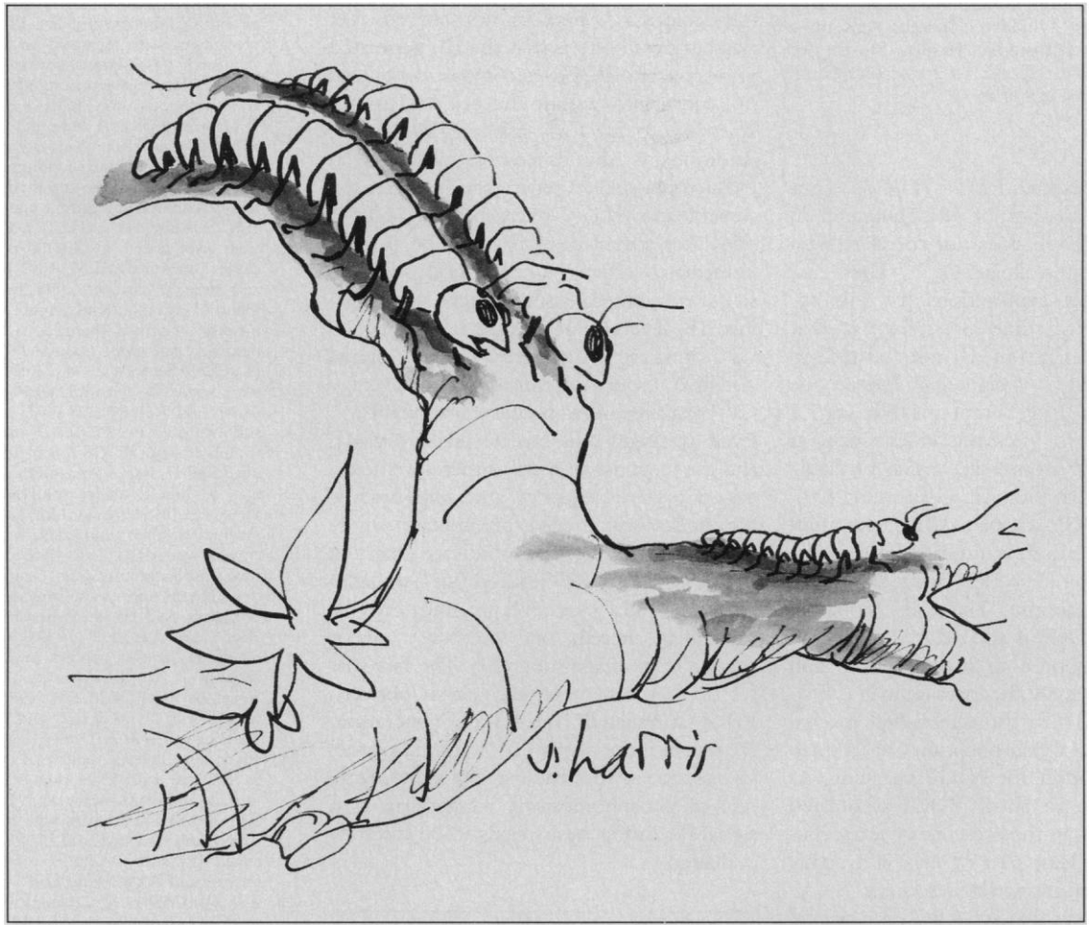

"I love to hear the pitter-patter-patter-patter-patter-patter-patter patter-patter-patter-patter-patter-patter-patter-patter-patter-patter-patter of his tiny feet."