early *Drosophila* embryos indicates that after nine cell divisions (512-cell stage; 90 min at 25°C) the primordial germline cells migrate to the posterior pole of the egg, are enveloped in a cell membrane, and are expelled from the syncytial mass of the egg [H. Steller and V. Pirrotta, *Dev. Biol.* **109**, 54 (1985)]. Some injected DNA becomes inserted into the germline at this time; that which remains in the cytoplasm slowly degrades. It occurred to us that behavioral transmission of P elements by mites, feeding at the posterior pole of fly eggs, is not an unrealistic possibility but, that because of the vagaries involved the likelihood of heuristically detecting such an event might be small.
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35. Controls: A metal spatula was used to sample culture medium from experimental fly bottles (approximately equivalent to the volume of 200 mites). Fly-debris controls contained culture medium plus at least one visible *Drosophila* body part (a portion of wing, leg, cuticle, and so forth) isolated from the experimental culture bottle. In both cases, the entire sample was used in a single PCR assay.
36. We thank E. E. Lindquist for identification of *P. regalis*, E. E. Lindquist and E. W. Baker for helpful discussions, M. Q. Benedict, W. A. Brown, A. C Cohen, L. D. Densmore, H. H. Hagedorn, G. W. Krantz, E. E. Lindquist, J. M. C. Ribeiro, and R. E. Strauss for comments on the manuscript, and M. J. Kaliszewski for samples of *P. longipilis* and *L. subterraneus* and for discussions. Supported by John D. and Katherine T. MacArthur Foundation vector grant 8900408 to the Center for Insect Science (grant to M.A.H. and M.G.K. and postdoctoral fellowship to J.B.C.), BARD grant IS-1397-87 to M.A.H. and NIH research grant 36715 to M.G.K.

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Inhibition of Neointimal Smooth Muscle Accumulation After Angioplasty by an Antibody to PDGF

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Approximately 30 to 40 percent of atherosclerotic coronary arteries treated by angioplasty or by bypass surgery occlude as a result of restenosis. This restenosis is due principally to the accumulation of neointimal smooth muscle cells, which is also a prominent feature of the advanced lesions of atherosclerosis. The factors responsible for the accumulation of intimal smooth muscle cells have not been identified. Platelet-derived growth factor (PDGF) is a potent smooth muscle chemoattractant and mitogen. It is present in platelets and can be formed by endothelium, smooth muscle, and monocyte-derived macrophages. The development of an intimal lesion in the carotid artery of athymic nude rats induced by intraarterial balloon catheter deendothelialization was inhibited by a polyclonal antibody to PDGF. These data demonstrate that endogenous PDGF is involved in the accumulation of neointimal smooth muscle cells associated with balloon injury and may be involved in restenosis after angioplasty, and perhaps in atherogenesis as well.

PDGF IS A POTENT MITOGEN AND chemoattractant for connective tissue cells (*1*). The PDGF family consists of dimeric molecules that can exist as homodimers or heterodimers of two distinct but related peptide chains termed PDGF-A and PDGF-B (*1*). PDGF is expressed at low or undetectable concentrations in normal adult tissues, but its expression is increased after tissue injury. Two distinct PDGF receptors have also been identified, one that binds either the A or the B chain (the α-subunit) and one that binds only the B chain (the β-subunit) (*2*). The capacity of cells such as smooth muscle to respond to PDGF may depend on the limited availability of appropriate receptors or the presence of particular dimeric forms of PDGF, or both. Investigations of experimentally induced atherosclerosis, naturally occurring human atherosclerosis, and smooth muscle accumulation associated with failure of vascular grafts have all demonstrated increased expression of PDGF and its receptors by Northern (RNA) blot analysis, in situ hybridization, and immunohistochemistry (*3, 4*). However, this association does not permit one to determine whether PDGF functions in the migration or proliferation of smooth muscle cells in lesions of atherosclerosis or graft restenosis. To determine whether PDGF is involved in these processes, we have examined an animal model of angioplasty, intraarterial balloon catheter deendothelialization of the rat carotid artery.

Deendothelialization with an intraarterial balloon catheter that dilates an artery induces injury to the innermost layers of medial smooth muscle and may even kill some of the innermost cells. This is followed by a round of proliferation of the medial smooth muscle cells, after which many of them migrate into the intima through fenestrae (natural openings) in the internal elastic lamina and subsequently proliferate to form a neointimal lesion (*5, 6*). In the rat, smooth muscle proliferation, as determined by the incorporation of [^{3}H]thymidine autoradiography, reaches a maximum in the medial layer of the artery 48 hours after ballooning and in the intima of the artery after 96 hours (*5*). Although smooth muscle proliferation can persist near the luminal surface as late as 12 weeks after balloon injury, the number of arterial smooth muscle cells does not increase after 2 weeks. The factors responsible for these events are not yet known.

Balloon catheter injury of the rat carotid artery induces the expression of mRNA for both PDGF-A chain and the PDGF receptor α- and β-subunits in the resulting neointimal lesion (*3*). In this study, we used a goat polyclonal antibody to PDGF (anti-PDGF) to examine the formation of intimal lesions in the carotid artery after balloon catheter deendothelialization. Anti-PDGF immunoglobulin G (IgG) was administered to athymic nude (nu/nu) rats (*7*) before and after balloon catheter deendothelialization of the carotid artery (*8*). We used athymic nude rats because of their inability to mount an immune response (*9*) to the large doses of antibody administered over 9 days. The neointimal response to balloon injury in the nude rat is essentially the same as that observed in other strains of rat (*10*).

We obtained the polyclonal antibody to human platelet PDGF by immunizing goats with PDGF prepared from human platelets (*11, 12*). The antibody was characterized for its ability to neutralize both chemotactic and mitogenic responses to PDGF in vitro before being used in vivo (*13*) (Fig. 1). The anti-PDGF IgG neutralizes the mitogenic activity of all dimeric forms of human PDGF (*13*) and has no direct effect on other

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known growth factors (*14*). The antibody specifically recognized a major protein band with a molecular size of 37,000 and a minor protein band of 35,000 on immunoblots of rat platelet releasate (a rich source of PDGF) (Fig. 1A). These bands are slightly larger in size than those of purified human PDGF (Fig. 1A). The anti-PDGF also completely neutralized the PDGF binding competitive activity present in rat whole blood serum at 50 μg/ml (Fig. 1B). Furthermore, it abrogated PDGF-induced [^{3}H]thymidine incorporation by rat smooth muscle cells and 50% of the mitogenic activity of rat platelet releasate (*10*). The anti-PDGF also inhibited chemotaxis of rat carotid smooth muscle cells to purified PDGF (*15*) and inhibited most of the chemotactic activity in rat platelet releasate (Fig. 1C). The major form of PDGF within rat platelets is the B-chain homodimer (*16*). PDGF-BB and rat platelet releasate were equally potent in inducing chemotaxis of rat smooth muscle cells and much more potent than transforming growth factor–β (TGF-β) or basic fibroblast growth factor (bFGF) (Fig. 1C). In contrast, the control medium, the nonimmune IgG, and PDGF-AA exhibited no chemotactic activity (Fig. 1C). The latter observation reflects the relatively low expression of PDGF receptor α-subunits by these cells (*10*).

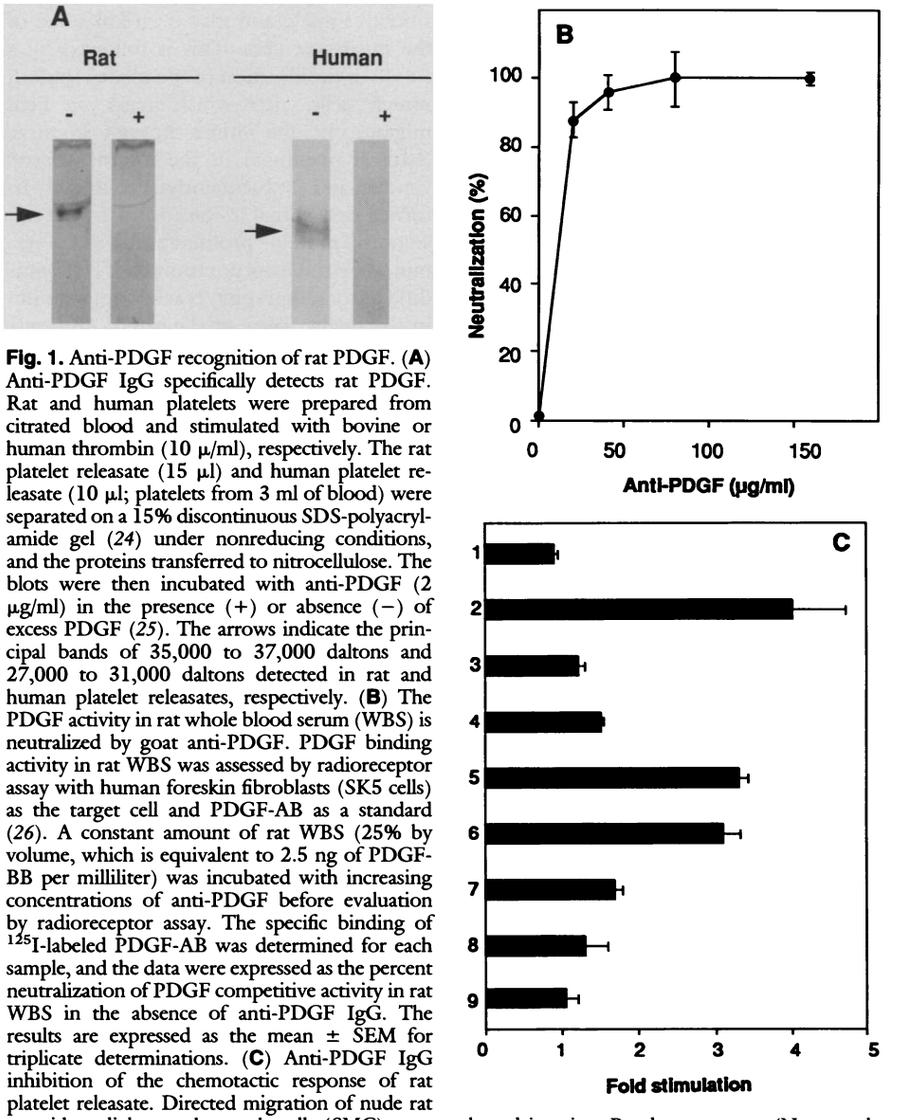

Fig. 1. Anti-PDGF recognition of rat PDGF. (**A**) Anti-PDGF IgG specifically detects rat PDGF. Rat and human platelets were prepared from citrated blood and stimulated with bovine or human thrombin (10 μ/ml), respectively. The rat platelet releasate (15 μl) and human platelet releasate (10 μl; platelets from 3 ml of blood) were separated on a 15% discontinuous SDS-polyacrylamide gel (*24*) under nonreducing conditions, and the proteins transferred to nitrocellulose. The blots were then incubated with anti-PDGF (2 μg/ml) in the presence (+) or absence (−) of excess PDGF (*25*). The arrows indicate the principal bands of 35,000 to 37,000 daltons and 27,000 to 31,000 daltons detected in rat and human platelet releasates, respectively. (**B**) The PDGF activity in rat whole blood serum (WBS) is neutralized by goat anti-PDGF. PDGF binding activity in rat WBS was assessed by radioreceptor assay with human foreskin fibroblasts (SK5 cells) as the target cell and PDGF-AB as a standard (*26*). A constant amount of rat WBS (25% by volume, which is equivalent to 2.5 ng of PDGF-BB per milliliter) was incubated with increasing concentrations of anti-PDGF before evaluation by radioreceptor assay. The specific binding of ^{125}I-labeled PDGF-AB was determined for each sample, and the data were expressed as the percent neutralization of PDGF competitive activity in rat WBS in the absence of anti-PDGF IgG. The results are expressed as the mean ± SEM for triplicate determinations. (**C**) Anti-PDGF IgG inhibition of the chemotactic response of rat platelet releasate. Directed migration of nude rat carotid medial smooth muscle cells (SMC) were evaluated in micro-Boyden apparatus (Neuroprobe Inc., Cabin John, MD). We determined the directed migration of the rat SMC after 4 hours at 37°C by counting the cells that migrated in response to different stimulants placed in the lower chamber as described (*10, 27*). Samples were evaluated in triplicate and included the following: 1, PDGF-AA (5 ng/ml); 2, PDGF-BB (10 ng/ml); 3, TGF-β (300 pg/ml); 4, bFGF (500 pg/ml); 5, rat platelet releasate (RPR); 6, RPR plus 500 μg of anti-PDGF per milliliter; 7, RPR plus 1 mg of anti-PDGF per milliliter; 8, nonimmune IgG, 1 mg/ml; and 9, control media. The concentrations selected for comparison were shown by separate experiments to induce a maximal response (*15*). Five high-power fields were counted for triplicate wells, a total of 15 fields per condition, and the results expressed as fold increase (mean ± SEM) in cell migration above a buffer control. In order to assess the effects of the antibodies on the chemotactic response, we incubated PDGF with the anti-PDGF for 30 min at 37°C before using it in the assay.

To determine dosage levels of the antibody in vivo, we evaluated the clearance of the anti-PDGF. Plasma antibody concentrations in the rats treated with the anti-PDGF determined by enzyme-linked immunosorbent assay (ELISA) (*17*) were reduced by 50% after 30 hours, and daily intraperitoneal administration of anti-PDGF IgG (60 mg per 100 g of body weight) maintained plasma concentrations of 1000 μg/ml during the 9-day duration of the experiment. At these concentrations, there was no significant effect on platelet counts or complement levels (*15*) and in vitro chemotaxis to rat platelet releasate was completely inhibited (Fig. 1C).

Administration of anti-PDGF before and after balloon catheter deendothelialization reduced the thickness and cellular content of the neointima (Fig. 2). Quantitative image analysis of the neointima of the 19 animals in each experimental group demonstrated that administration of anti-PDGF resulted in a 40.9% reduction in the area of the neointima ($P < 0.01$ by two-tailed t test) (Fig. 3).

We prepared representative sections of each carotid artery to evaluate labeling indices (*18*). [^{3}H]thymidine was administered 17, 9, and 1 hour before the animals were killed to label proliferating cells. The mean thymidine labeling index of the medial cells 8 days after injury is 4.39 ± 1.02%, and that of the neointimal cells is 27.3 ± 2.31%. Corresponding values for the anti-PDGF group are 4.67 ± 0.79% for the medial cells and 31.2 ± 1.94% for the neointimal cells. However, there are no statistically significant differences in either the medial or intimal labeling indices between the anti-PDGF–treated rats and the nonimmune IgG–treated rats. These data suggest that in this time frame and for the dose of anti-PDGF administered the decrease in intimal area is due primarily to interference with PDGF-induced chemotactic migration of medial smooth muscle cells into the intima. In the absence of data concerning the actual amount of antibody exposure at the cellular level to the individual smooth muscle cells in the different layers of the neointima and the media, we cannot exclude the possibility that larger amounts of anti-PDGF might have reduced smooth muscle replication as well as chemotaxis. This is of concern with regard to the neutralization of autocrine

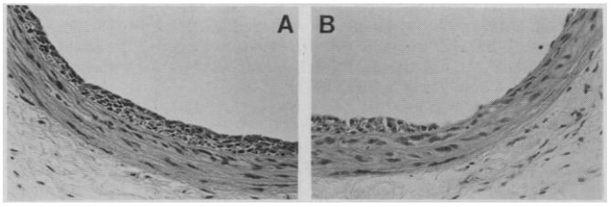

Fig. 2. Anti-PDGF inhibits the accumulation of intimal smooth muscle cells 8 days after balloon catheter injury. Hematoxylin- and eosin-stained cross sections of balloon-catheterized rat common carotid arteries taken 8 days after surgery from animals that were treated daily with either nonimmune goat IgG (**A**) or anti-PDGF IgG (**B**). Magnification ×400.

PDGF-AA, which may be produced by the smooth muscle cells in the balloon-treated carotid. PDGF-A chain mRNA, but not PDGF-B chain mRNA, is transiently and focally increased in the intimal smooth muscle cells after balloon injury (*3*). In in vitro studies, large amounts of anti-PDGF (5 mg/ml) are required to inhibit the mitogenesis that results from endogenously produced PDGF-AA induced by interleukin-1 or TGF-β (*13, 19*). In contrast, 0.8 mg of anti-PDGF per milliliter inhibited exogenously added PDGF-AA, whereas only 0.1 mg/ml was required to inhibit exogenously added PDGF-AB or PDGF-BB. Thus, it is difficult to know the required antibody level that would be effective in vivo.

These observations provide direct evidence that endogenous PDGF functions in vivo in the accumulation of smooth muscle cells that occurs after balloon catheter–induced injury. A single injection of an antibody to FGF inhibits (≈80%) the first round of replication of medial smooth muscle that occurs 24 to 48 hours after injury induced by a dilating balloon catheter but does not influence the size of the resultant intimal lesion (*20*). In contrast, in rats depleted of circulating platelets, no effect is observed on maximal smooth muscle replication in the media 2 days after balloon injury, but after 4 and 7 days there is a marked reduction in the size of the neointima (*21*). The latter studies suggest that substances released from platelets are primarily responsible for the movement of smooth muscle cells from the media into the intima. Our studies support these observations because the absence of a difference in [^{3}H]thymidine incorporation in the intima or the media of the artery after administration of either anti-PDGF or nonimmune IgG suggests that PDGF mediates its action principally by stimulating chemotactic migration of the smooth muscle cells into the intima.

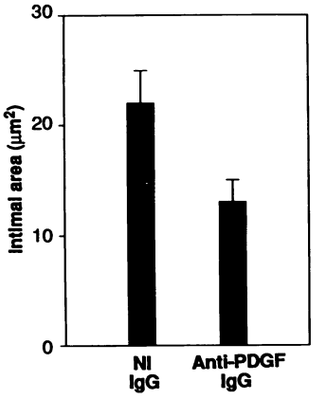

Fig. 3. Effect of anti-PDGF IgG on the accumulation of intimal smooth muscle cells after balloon catheter injury. The intimal cross-sectional areas of 19 animals treated with anti-PDGF and 19 animals treated with nonimmune (NI) IgG, 18 hours before injury and daily for 8 days after injury, were measured with an image-analysis system consisting of a Leitz microscope fitted with a ×25 objective (Leitz, Wetzlar, Germany) and a Video image-analysis system (Ai Cambridge, Papworth, Cambridge, U.K.). Cross-sectional areas from the left common carotid artery deendothelialized with a balloon catheter (*5*) were evaluated. Measurements were made in duplicate on three midcarotid sections, and the intimal areas were calculated for each rat. The mean ± SEM are plotted.

In this in vivo system PDGF appears to act largely by stimulating smooth muscle migration. This is further supported by the observation that continuous intravenous infusion of PDGF-BB for 7 days (40 μg/day per rat) to a rat injured with a filament loop catheter resulted in a 15-fold increase in the intimal lesion area (*22*). Analysis of autoradiograms after continuous infusion of [^{3}H]thymidine demonstrated a 21-fold increase in the number of unlabeled cells in the neointima of PDGF-BB–treated animals with no change in the total number of nondividing cells in the intima plus media. Although the [^{3}H]thymidine labeling of medial smooth muscle cells did increase fourfold in the PDGF-treated animals, the principal effect of PDGF-BB on the formation of intimal lesions was stimulation of the migration of medial smooth muscle cells into the intima.

Chemotactic migration of medial smooth muscle cells into the intima is a critical step in the development of the neointimal arterial lesion that results from balloon angioplasty. Our results suggest potential approaches to prevention of clinical restenosis, which is responsible for the failure of the occluded arteries treated by angioplasty (*23*), endarterectomy, or bypass surgery.

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replaced with paraformaldehyde (4%) in isotonic phosphate-buffered saline (PBS) (pH 7.4). After fixation in situ, the carotids were isolated and dissected free of adherent fat and connective tissue. Mid-carotid segments were embedded in paraffin for sectioning.
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12. Injections (six) of purified human PDGF (75 μg) were administered to a goat subcutaneously every 2 weeks for 3 months. The initial injection was in complete Freund's adjuvant with subsequent injections in incomplete Freund's adjuvant. Once a titer was established, plasma was collected by plasmapheresis every week, with periodic boosts of PDGF (300 μg) in incomplete Freund's. The IgG fraction was prepared by sodium sulfate precipitation and DEAE-Sephacel chromatography. The 18% sodium sulfate precipitate was resolubilized and dialyzed to remove excess sodium sulfate, and the dialysate was further purified by passage over a DEAE-Sephacel (Pharmacia, Piscataway, NJ) column. Purified IgG was obtained by elution with 0.01M phosphate buffer (pH 6.8). The pooled void volume and pH 6.8 wash were concentrated by ultrafiltration (PM-10, Amicon Corp., Danvers, MA) and dialyzed against PBS to a protein concentration of 60 to 100 mg/ml as determined by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall [*J. Biol. Chem.* **193**, 265 (1951)], with bovine serum albumin (BSA) as a standard and after application of a correction factor for immunoglobulin [J. A. Klosse, P. K. De Bree, S. K. Wadman, *Clin. Chim. Acta* **32**, 321 (1971)]. Before use, the IgG was filter-sterilized through a 0.22-μm filter (Millipore, Bedford, MA) and stored at 4°C. A nonimmune goat IgG was prepared by the same method with commercially available goat plasma.
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17. Microtiter plates (96-well, Nunc, Denmark) were coated with PDGF-AB (10 ng per well) for 18 hours at 4°C. Nonspecific protein binding was reduced by blocking with BSA (1%) in Tween 20 (0.2%) and PBS for 1 hour at 37°C. Dilutions of standards and samples were prepared in a 1:16 mixture of rat plasma–derived serum and PTB (0.05% Tween 20 and 0.2% BSA in PBS). Aliquots (100 μl) of sample or standards of known concentrations of anti-PDGF were incubated in wells for 90 min at 37°C. The plates were rinsed five times in wash buffer (0.5% Tween 20, 0.9% saline) and then incubated with 1:1000 biotinylated swine antibody to goat IgG (100 μl per well) (Caltag Laboratories, South San Francisco, CA) in PTB for 60 min at 37°C. Unbound secondary antibody was removed by rinsing in wash buffer and subsequently incubated with avidin and biotinylated peroxidase (Vector Labs, Burlingame, CA) for 30 min at 37°C. After washing, substrate (0.04% *o*-phenylenediamine, Sigma) was dissolved in a mixture of 0.05M citrate and 0.1M Na_2PO_4 (pH 5.0) that contained hydrogen peroxide (10 μl of a 30% solution per 25 ml); 100 μl was added to each well and incubated for 10 min at room temperature. The reaction was terminated by the addition of sulfuric acid (4N), and the absorbance of the reaction product was read at 490 nm.
18. Sections of carotid were deparaffinized and dipped in NTB-2 photograph emulsion (Kodak, Rochester, NY). The autoradiographs were exposed for 14 days at 4°C in a light-tight box and were then developed with Kodak D-19 developer and fixed with Kodak Rapid Fix. Cell nuclei were stained with hematoxylin. Cells with more than five silver grains above their nuclei were considered positive. We examined duplicate sections from two levels of each carotid under oil immersion, using a Zeiss photomicroscope with a ×63 objective (Carl Zeiss Inc., West Germany). Approximately 600 cells per well compartment were counted to determine the proportion of labeled cells.
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Regulation of Kainate Receptors by cAMP-Dependent Protein Kinase and Phosphatases

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In the mammalian central nervous system, receptors for excitatory amino acid neurotransmitters such as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–kainate receptor mediate a large fraction of excitatory transmission. Currents induced by activation of the AMPA-kainate receptor were potentiated by agents that specifically stimulate adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase A (PKA) activity or were supported by intracellular application of the catalytic subunit of PKA by itself or in combination with cAMP. Furthermore, depression of these currents by a competitive inhibitor of PKA indicates that AMPA-kainate receptors are regulated by endogenous PKA. Endogenous protein phosphatases also regulate these receptors because an inhibitor of cellular phosphatases enhanced kainate currents. Modulation of PKA and phosphatases may regulate the function of these receptors and thus contribute to synaptic plasticity in hippocampal neurons.

EXCITATORY AMINO ACID RECEPTORS, which contain ion channels, can be broadly classified as *N*-methyl-D-aspartate (NMDA) or non-NMDA receptors on the basis of selective pharmacological antagonism (*1*). Non-NMDA receptors have been further subdivided into AMPA and kainate subtypes although it has been difficult to find a specific antagonist for either subtype. Kainate currents do not desensitize, whereas AMPA currents desensitize rapidly (*2*). However, desensitization is not necessarily indicative of distinct receptor subtypes (*3*).

We have investigated the regulation of AMPA-kainate receptors by protein kinases (*4*) and phosphatases in mammalian neurons (*5*). We used kainate (Fig. 1) to avoid the desensitization that occurs with AMPA (*2, 3*). The amplitude of kainate currents declined exponentially with a time constant of 7 min when electrode solutions without adenosine triphosphate (ATP) were used (Fig. 1, A and C). This "washout" (*6*) of kainate currents was similar to that reported for NMDA currents (7), but it had a slower time course. Introduction of an ATP-regenerating solution (*8*) into the neurons by means of the recording electrode significantly reduced the washout of kainate currents (Fig. 1, B and C). In contrast, when we avoided intracellular dialysis by recording whole-cell currents with the nystatin patch technique (*9*), kainate currents were stable for periods of up to 2 hours after the initial period of patch perforation (Fig. 1D). Such evidence suggests that the magnitude of kainate currents is determined at least in part by the ability of the neuron to maintain intracellular phosphorylation.

In order to examine a possible role of PKA in the regulation of AMPA-kainate receptors, we quantified the effects of cAMP and the catalytic subunit of PKA (Sigma) on the washout of kainate currents. This washout was significantly retarded when the catalytic subunit of PKA (20 μg/ml) was included in the recording pipette. Intracellular applications of cAMP (40 μM) were associated with a transient potentiation of kainate currents. In contrast, cAMP did not enhance NMDA currents (Fig. 1E). A combination of ATP, cAMP, and PKA was the most efficacious in retarding or preventing washout of kainate currents.

The recording electrode was internally perfused with various solutions (*10*). The patch pipette initially contained a control

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