that only antisera against ALK-5 immunoprecipitated cross-linked TGF-β type I receptor complexes, and antisera against ALK-2 and ALK-4 immunoprecipitated cross-linked activin type I receptor complexes. Moreover, among the six ALKs, only ALK-5 efficiently restored the TGF-β activation of PAI-1 synthesis in mutant Mv1Lu cells.

The low efficiency binding of TGF-β to ALKs other than ALK-5 and of activin to ALKs other than ALK-2 and ALK-4, when expressed together with type II receptors in COS-1 cells, appears not to represent true physiological interactions. The possibilities remain, however, that other ALKs may bind TGF-β2 or TGF-β3 or transduce TGF-β or activin signals other than PAI-1 induction. Differences may also prevail between different cell types. Alternatively, the other ALKs might be signaling receptors for other ligands in the TGF-β superfamily, such as bone morphogenetic proteins or Müllerian inhibiting substance, in combination with other type II receptors.

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24. A λEX*lox* cDNA library from 12-day mouse embryos (Novagen) was screened with a ^{32}P-labeled probe, 11.1 (*7, 8*), which corresponds to a part of the kinase domain of human ALK-4. Hybridization to nitrocellulose replica filters and purification of bacteriophage were done as described (*17*). The filters were washed two times with 2 × standard saline citrate (SSC) (1 × SSC is 15 mM sodium citrate and 150 mM NaCl) and 0.1% SDS at 37°C for 15 min and two times with 0.5 × SSC and 0.1% SDS at 55°C for 15 min. Plasmids were excised from λEX*lox* following the manufacturer's protocol. Nucleotide sequencing was done on both strands with Sequenase (U.S. Biochemical Corporation) and specific oligonucleotide primers. Among 20 clones obtained, 4 clones were identical to each other that had novel sequences and termed ALK-6.
25. We generated transient expression plasmids encoding ALK-1 through -6, TβR-II, and ActR-II by subcloning into the pSV7d expression vector (*18*) or into the pcDNA I expression vector (Invitrogen). For transient transfection, COS-1 cells (American Type Culture Collection) were transfected with plasmids (10 μg) by a calcium phosphate precipitation method with a mammalian transfection kit (Stratagene), following the manufacturer's protocol. Recombinant human TGF-β1 and activin A were iodinated with the chloramine T method (*6, 19*). Cross-linking and immunoprecipitation were done as described (*8*). The samples were analyzed by SDS-PAGE and autoradiography.
26. Rabbit antisera to ALK-5 and TβR-II were made against the intracellular juxtamembrane part of ALK-5 and the COOH-terminal part of TβR-II, respectively (*8*). An antiserum to ActR-II was prepared against the COOH-terminal part of the ActR-II protein (*20*). Antisera against ALKs 1, 2, 3, 4, and 6 were raised against synthetic peptides corresponding to the amino acid sequences of the intracellular juxtamembrane parts of ALKs: ALK-1 peptide, amino acids 145 to 166; ALK-2 peptide, amino acids 151 to 172; ALK-3 peptide, amino acids 181 to 202; ALK-4 peptide, amino acids 153 to 171; and ALK-6 peptide, amino acids 151 to 168. The peptides were coupled to keyhole limpet hemocyanin (Calbiochem-Behring) with glutaraldehyde (*21*), mixed with Freund's adjuvant, and used to immunize rabbits.
27. The R mutant of Mv1Lu cells (clone 4-2) was transfected with plasmids (10 μg) containing cDNAs for ALKs or a control plasmid by the calcium phosphate precipitation method. Transfected cells were incubated with or without TGF-β1 (16 ng/ml) for 2 hours in serum-free MCDB 104 medium without methionine. Cultures were then labeled with [^{35}S]methionine (40 μCi/ml) for 2 hours. Extracellular matrix proteins were prepared as described (*22*) and analyzed by SDS-PAGE and then by fluorography with Amplify (Amersham).
28. For the generation of PAE–Chim A cells, a plasmid (chim A) containing the extracellular domain and COOH-terminal tail of ActR-II (amino acids −19 to 116 and 465 to 494, respectively) (*6*) and the kinase domain of TβR-II (amino acids 160 to 543) (*5*) was constructed and transferred into pcDNA I/neo (Invitrogen). PAE cells were stably transfected with the chim A plasmid by electroporation, and cells expressing the chim A protein were established as described (*8*).

29. We thank Y. Eto (Ajinomoto Company, Inc., Japan) for providing activin A, H. Ohashi (Kirin Brewery Company, Ltd., Japan) for TGF-β1, K. Alitalo for a mouse cDNA library, L. Mathews and W. W. Vale for mouse ActR-II cDNA, D. Huylebroeck for valuable discussion, A. Morén and S. Grimsby for technical assistance, and U. Engström and C. Wernstedt for preparing the synthetic peptides and oligonucleotides, respectively. Supported by a European Molecular Biology Organization postdoctoral fellowship (P.t.D.) and Japanese Eye Bank Association (H.Y.).

22 October 1993; accepted 23 February 1994

FGF-2: Apical Ectodermal Ridge Growth Signal for Chick Limb Development

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The apical ectodermal ridge permits growth and elongation of amniote limb buds; removal causes rapid changes in mesodermal gene expression, patterned cell death, and truncation of the limb. Ectopic fibroblast growth factor (FGF)–2 supplied to the chick apical bud mesoderm after ridge removal will sustain normal gene expression and cell viability, and allow relatively normal limb development. A bioassay for FGFs demonstrated that FGF-2 was the only detectable FGF in chick limb bud extracts. By distribution and bioactivity, FGF-2 is the prime candidate for the chick limb bud apical ridge growth signal.

The developing chick limb serves as an experimental system to understand pattern formation (*1*). The limb bud is composed of mesoderm and overlying ectoderm. Capping nearly the entire apex of the bud is a pseudostratified columnar epithelium called the apical ectodermal ridge, which is spatially limited by the simple cuboidal dorsal and ventral limb bud epithelia (*2*). If the ridge is removed surgically it fails to regenerate. Subsequently, only those parts of the limb already determined will develop (*3*). It has been proposed that the ridge, in a permissive way and without direct cell contact (*4*), maintains the mesodermal cells about 200 μm beneath it in a rapidly proliferating and undifferentiated region called the progress zone (*5*). When cells

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leave this zone of ridge influence they begin to differentiate according to their position in the limb. Fibroblast growth factors (FGFs) are prime candidates as possible ridge growth factors because they have yielded provocative results in tissue culture studies (for example, 6).

There are nine members of the fibroblast growth factor family (7). FGF-2 and FGF-4 were used in two in vivo studies of chick limb development. Ectopic production of human or bovine FGF-2 in the chick limb bud in vivo by a replication-defective retroviral vector caused patterned extra skeletal elements to form (8). When ectopic human recombinant FGF-4 was supplied to the chick limb bud after ridge removal, development continued and there was relatively normal formation of the limb skeleton (9). The distribution of FGF-2 transcripts and protein in the chick ridge and mesoderm (10) and its effect when ectopically expressed in vivo (8) suggest FGF-2 affects limb growth and is a prime candidate for the growth signal secreted by the chick ridge.

To test this we provided ectopic FGF-2 after apical ridge removal from stage 20 (day 3) chick embryo wing buds by immediately implanting an Affi-gel blue bead pre-adsorbed with human recombinant FGF-2 into the progress zone (11) and allowing development until day 10. The shapes of all limb buds were recorded by drawing with a camera lucida to have a precise record of the outline of the bud and the placement of the bead. Control limbs were truncated at the level of the humerus (Fig. 1, A and B, and Table 1). When a single FGF-2 adsorbed bead was grafted within the progress zone of a bud after ridge removal, development continued beyond the elbow joint; 9 of 13 limbs showed development to the wrist (Fig. 1, C and D, and Table 1). Histological sections through the apex of the buds ruled out regeneration of the ridge under the influence of FGF-2 [compare with (12)].

We examined the kinetics of release of iodinated FGF-2 from Affi-gel blue beads in the limb bud (13). About half of the total iodinated FGF-2 loaded into the bead was released in the first 24 hours (Fig. 2). The approximate amount of FGF-2 released into the limb bud for the first 24 hours is 1.2 pmol and the second 24 hours is 0.6 pmol (14). This suggested the amount of FGF-2 remaining in the implanted bead dropped below a critical level after 24 hours. To sustain FGF-2 release, 24 hours after placement of the first bead we grafted a second FGF-2 bead into the progress zone distal to the first one. Virtually complete limbs formed from all limb buds (n = 14) receiving two sequentially placed beads (Fig. 1, E to G, and Tables 1 and 2). Most beads were grafted to the midline of the bud; however, there was no difference in the result when the bead was grafted either anterior or posterior to the midline.

Histological sections showed that the patterned cell death of progress zone cells that peaks 8 hours after ridge removal (12) failed to occur in the presence of FGF-2. Cell death was not eliminated completely, but the massive, patterned death of progress zone cells was not observed. Three hours after ridge removal, control progress zone cells lose detectable expression of the homeobox-containing gene *Msx-1* (Fig. 1H) [compare with (15)], whereas FGF-2 permitted the progress zone cells to maintain

Table 1. Effects of apical ridge removal (AER$^-$) at stage 20, with or without addition of FGF-2. Number (percentage) of limbs that were truncated at the indicated extent of development.

Treatment	Limb truncation			
	Humerus	Humerus+	Radius/Ulna	Digits
AER$^-$	10 (83%)	2 (17%)*	0	0
AER$^-$ and PBS bead	5 (100%)	0	0	0
AER$^-$ and FGF-2 bead	0	4 (31%)†	9 (69%)	0
AER$^-$ and two FGF-2 beads	0	0	0	14‡

*These two embryos had a very small cartilage nodule beyond humerus but were stage 20+ when the apical ridge was removed. †One embryo had part of a radius, one had bent radius and short ulna, one embryo had a radius but no ulna, and one had complete but shortened radius and ulna. ‡See Table 2 for details.

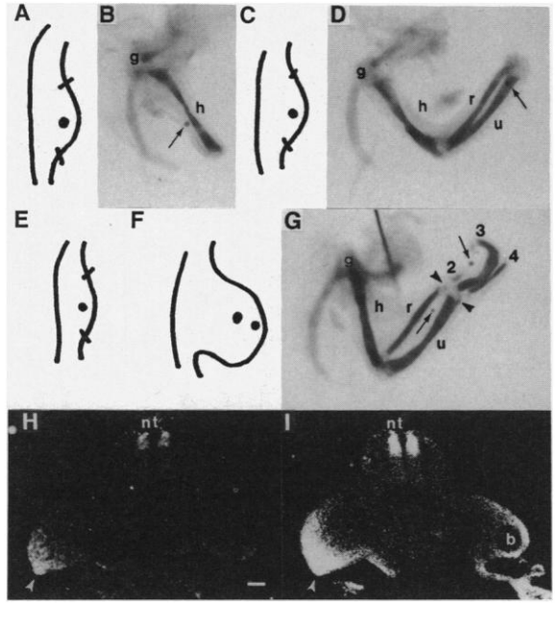

Fig. 1. Effects of apical ridge removal at stage 20, with or without addition of FGF-2. (**A**) Camera lucida drawing of stage-20 limb after ridge removal and a phosphate-buffered saline bead implanted into the progress zone. The two short lines indicate the anterior to posterior extent of ridge removal. (**B**) Limb that formed from the bud drawn in (A) is truncated at the distal end of humerus (h). Arrow indicates the bead; g indicates shoulder girdle. (**C**) Camera lucida drawing of stage 20 limb bud after apical ridge was removed and an FGF-2 bead implanted into the progress zone. (**D**) Limb that formed from bud drawn in (C) developed to the wrist with normal appearing humerus (h), radius (r), and ulna (u); carpal elements and digits are missing. (**E**) Camera lucida drawing of early stage-20 limb bud after ridge removal and grafting of an FGF-2 bead. (**F**) Camera lucida drawing of bud drawn in (E), 24 hours later, after grafting a second FGF-2 bead distal to the first. The outline of the limb bud is normal. (**G**) Limb formed from the bud in (E) and (F) shows distally complete development with humerus, radius, ulna, carpal elements (arrowheads), a partial digit 2, and digits 3 and 4. Digits 3 and 4 are not normal, but are discrete and recognizable. See figure 2E in (8) for normal chick wing skeleton. (**H** and **I**) Expression of *Msx-1* gene in wing buds 3 hours after ridge removal (right wing buds) and grafting a single bead with PBS (H) or FGF-2 (I). Left wing buds are normal controls: b, bead; arrowheads, apical ridge; nt, neural tube; bar represents 100 μm. The sections were hybridized with the *Msx-1* probe as described (15). The *Msx-1* probe was derived from the 3′ untranslated region of the chick *Msx-1* gene.

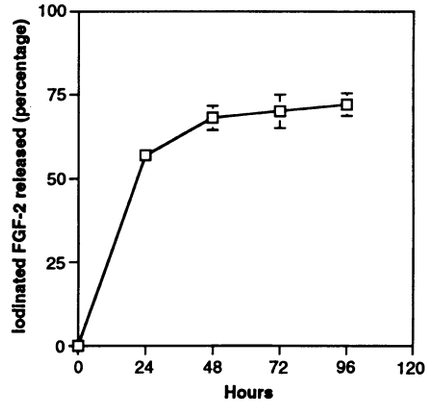

Fig. 2. Release of iodinated FGF-2 expressed as percentage released from the bead after grafting to a stage 20 limb bud. Each point represents five grafted beads. Error bars indicate standard deviation.

expression of *Msx-1* (Fig. 1I). If the bead was left behind by continued growth, only the progress zone cells showed *Msx-1* transcripts; the cells around the bead at 12 to 24 hours had background levels of *Msx-1*. This is a puzzling observation that requires further investigation. We examined the expression of the most 5′ members of the *Hoxd* cluster of homeobox genes, whose patterns of expression are dependent on continued signaling by the ridge (*16*). We found that *Hoxd-12* and *Hoxd-13* expression continued 18 to 24 hours after ridge removal, and that, similar to previous reports, there appeared to be less expression of *Hoxd-13* in progress zone cells after ridge removal (*16*) compared with unoperated limbs or with FGF-2 bead grafts.

FGF-2 will maintain the viability, normal gene expression, growth, and differentiation of chick limb bud mesoderm and replace the apical ridge. A similar case was made for FGF-4 (9). Chicken-specific FGF-4 probes are not available to assess directly the distribution of FGF-4 in the chick limb bud, as has been done for FGF-2 (*10*). To gain insights into whether both growth factors are present in the stage 20 wing bud, we used a quantitative bioassay that relies on FGF-mediated repression of differentiation in a mouse myoblast cell line (MM14) capable of detecting as little as 0.3 fmol/ml (*17*). To distinguish the activity of FGF-2 from FGF-4 in extracts of limb mesoderm and ectoderm, we used a monoclonal antibody specific to FGF-2 (anti–FGF-2) (*10*) that eliminated repression of differentiation activity on MM14 cells by human recombinant FGF-2; the antibody did not block repression of differentiation mediated by human recombinant FGF-4 (Fig. 3A). After treatment with anti–FGF-2, FGF activity in limb mesoderm and ectoderm extracts was reduced by 94% and 97%, respectively, compared with untreated extracts (Fig. 3B).

These observations raise a number of questions. For example, it is striking that ectopic release of FGF-2 after ridge removal does not cause a featureless lump or tumor-like growth on the limb due to uncontrolled proliferation; patterning of the limb was normal. The ridge signal does not need to come from the most apical part of the bud, as indicated by the results after the bead is grafted within the progress zone where half or even most of the responding cells receive the signal 180° from the normal in vivo position. Therefore, a distal-to-proximal orientation of the ridge signal is not necessary for normal limb bud outgrowth. Furthermore, during stage 21 the posterior limb bud mesoderm grows faster than the anterior limb bud mesoderm. This continues at subsequent stages, with the result that most of the limb develops from the posterior mesoderm (*18*). This pattern of limb development is also seen after ridge removal followed by grafting two FGF-2 beads 24 hours apart (Fig. 1G). It is likely that the factors that cause the posterior mesoderm to become predominant in the bud, and that cause the bud to elongate, are already in place at stage 20; FGF-2 simply maintains them. This fits well with the notion that the ridge signal is a permissive inducer. A straightforward interpretation of the data is that, after ridge removal, ectopic FGF-2 maintains the cells of the progress zone in a healthy and proliferative condition. This is consistent with the normal distribution of FGF-2 in the chick limb bud (*10*).

The effect of ridge removal on limb development was first published in 1948 (*3*). Subsequently, numerous reports have framed important questions on how the ridge carries out its unique function. One such question is, what is the ridge signal? There are three generally accepted criteria to establish a signaling substance between cells or tissues (*19*). We have established two of these for FGF-2. First, FGF-2 has the correct distribution in the chick limb bud to be the signaling molecule and FGF-2 from the limb bud is active in a biological assay. Second, when the ridge is removed, ectopic FGF-2 will replace ridge function at all levels of organization. The third criterion, namely inhibition of the signaling substance, is yet to be fulfilled.

In this report we demonstrate that the application of a single factor can permit relatively complete limb development in vivo after removal of the apical ectodermal ridge. By distribution and biological activity, FGF-2 emerges as the prime candidate to be the chick apical ridge signaling growth factor.

Table 2. Cartilage elements present at day 10 of incubation in limbs that had received FGF-2 beads 24 hours apart following apical ridge removal at stage 20. The carpal elements require stages 21 to 25, days 3.5 to 4.5, to be determined (*5*). After 24 hours the second bead would retain 50% or less of FGF-2 and may have dropped below a critical threshold for progress zone maintenance. This is mirrored in the gradual decline in the development of the most distal elements of the manus. Metacarpals are included with the digits for simplicity of presentation.

Element	Limbs	
	(*n*)	(%)
Humerus	14	100
Radius	14	100
Ulna	14	100
Olecranon process	14	100
Carpal elements		
Radiale	14	100
Carpal	14	100
Ulnare	14	100
Digit 2		
Metacarpal 2	9*	64
Phalangeal element 1	7	50
Phalangeal element 2	3	21
Digit 3		
Metacarpal 3	13†	93
Phalangeal element 1	9‡	64
Phalangeal element 2	2*	14
Digit 4		
Metacarpal 4	11§	79
Phalangeal element 1	2*	14

*1, †4, ‡2, or §5 individual elements were fused with another element.

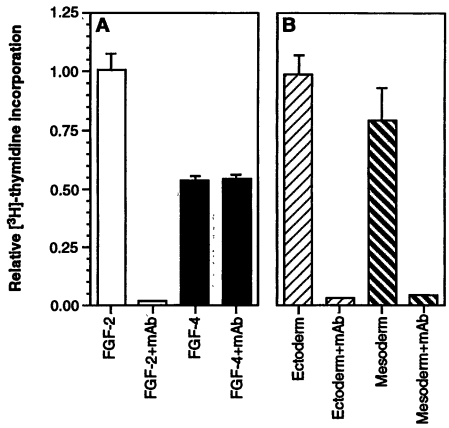

Fig. 3. Monoclonal antibody to FGF-2 (mAb) blocks repression of skeletal muscle cell (MM14) differentiation mediated by FGF-2 or by activity present in limb tissue extracts. MM14 differentiation was assayed by measurement of exit from the cell cycle (*17*). (**A**) [³H]Thymidine incorporation by MM14 cells incubated with human recombinant FGF-2 (open bars) (n = 3) and human recombinant FGF-4 (filled bars) (n = 3); antibody treatment, n = 2. (**B**) MM14 cells were incubated with ectodermal (thin diagonal bars) or mesodermal (thick diagonal bars) extracts to produce maximal activity for each extract in the assay (n = 3). Addition of anti-FGF-2 monoclonal antibody, n = 2. Error bars are standard error.

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reports are appearing that FGF-2 may cause up-regulation of its own message and protein, at least in cells in culture [J. C. Fox and J. L. Swain, *In Vitro Cell. Dev. Biol.* **29A**, 228 (1993); B. Flott-Rahmel *et al.*, *Neuroreport* **3**, 1077 (1992)]. This must be explored further in the limb bud.

11. Fertile White Leghorn chicken eggs were incubated at 38°C for 3 days [stage 20, V. Hamburger and H. L. Hamilton, *J. Morphol.* **88**, 49 (1952)]. After staining with Nile blue, the apical ridge was removed with a tungsten needle. Controls were either reincubated without further treatment or received an Affi-gel blue bead soaked in phosphate-buffered saline (PBS). FGF-2–loaded beads were grafted immediately following ridge removal. Affi-gel blue beads (Bio-Rad) 200 μm in diameter were incubated in either PBS or 0.56 nmol human recombinant FGF-2 for 1 hour at 37°C.
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13. Human recombinant FGF-2 was iodinated as described [B. B. Olwin and S. D. Hauschka, *J. Cell Biol.* **110**, 503 (1990)] except that ^{125}I-labeled FGF-2 was not purified from free ^{125}I, and no carrier protein was used. Affi-gel blue beads were incubated in iodinated FGF-2 (0.57 μg, 6.1 × 10^5 cpm/μl) for 1 hour at 37°C, agitating every 10 min. Beads were rinsed and counted individually in a Wallac Clinigamma counter for 1 min. Selected beads were implanted into the right wing bud of stage 20 embryos, which were then reincubated for 24, 48, 72, or 96 hours, when the bead was removed from the wing and the amount of radioactivity in the bead determined.
14. Forty 200-μm Affi-gel blue beads incubated in iodinated FGF-2 (32 pmol in 100 μl) adsorbed 18% of the iodinated FGF-2, yielding 0.14 pmol/bead (2 × 10^5 cpm per bead). Incubation of 40 beads in 0.23 pmol unlabeled FGF-2 containing iodinated FGF-2 yielded a nearly identical incorporation of 19%. It is unlikely the beads are saturated and thus they will incorporate similar amounts of FGF-2 at low concentrations (30 pmol) used to examine release in vivo or higher concentrations (0.6 nmol) used to replace apical ridge function. Incubation of beads in 1 ml of 0.56 mmol unlabeled FGF-2 then yields 2.4 pmol loaded per bead [compare with A. Hayek *et al.*, *Biochem. Biophys. Res. Comm.* **147**, 876 (1987)].
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26 October 1993, accepted 4 March 1994

Roles of N-Type and Q-Type Ca^{2+} Channels in Supporting Hippocampal Synaptic Transmission

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Several types of calcium channels found in the central nervous system are possible participants in triggering neurotransmitter release. Synaptic transmission between hippocampal CA3 and CA1 neurons was mediated by N-type calcium channels, together with calcium channels whose pharmacology differs from that of L- and P-type channels but resembles that of the Q-type channel encoded by the α_{1A} subunit gene. Blockade of either population of channels strongly increased enhancement of synaptic transmission with repetitive stimuli. Even after complete blockade of N-type channels, transmission was strongly modulated by stimulation of neurotransmitter receptors or protein kinase C. These findings suggest a role for α_{1A} subunits in synaptic transmission and support the idea that neurotransmitter release may depend on multiple types of calcium channels under physiological conditions.

Electrophysiological studies have defined several pharmacologically distinct high voltage–activated (HVA) Ca^{2+} channels on neuronal cell bodies (*1*). Among these the L-, N-, and P-type channels are the best known (*1*), but other classes of channels have been revealed by molecular cloning and electrophysiology (*2–7*). How diverse Ca^{2+} channels contribute to synaptic transmission in the central nervous system is not entirely clear (*7*). We studied the synapse between hippocampal CA3 and CA1 neurons (*8*), a focus of interest in examination of glutamatergic transmission and synaptic plasticity (*9*). At this synapse, inhibition of L-type channels by nifedipine has little effect (*10*), and much of the transmission remains after blockade of N-type channels by ω-Conotoxin GVIA (ω-CTx-GVIA) (*10, 11*). We found that the Ca^{2+} channels that mediate the remaining transmission are pharmacologically distinct from classical L- and P-type Ca^{2+} channels. Instead, their pharmacological profile resembled that of α_{1A} Ca^{2+} channel subunits expressed in *Xenopus* oocytes (*4*) and the Q-type Ca^{2+} channel current in cerebellar granule neurons (*6*). Stimulation of neurotransmitter receptors can greatly attenuate synaptic transmission mediated by α_{1A} channels. Reductions in the contribution of either Q- or N-type channels greatly increased the degree to which closely spaced stimuli facilitated synaptic transmission. These results suggest that cooperation may occur among multiple Ca^{2+} channel types in the control of transmitter release and may be advantageous for precise regulation of the strength- or frequency-dependence of synaptic function.

Selective blockade of N-type Ca^{2+} channels with 1 μM ω-CTx-GVIA (*12*) caused a rapid but incomplete depression of synaptic transmission (Fig. 1A), which was not reversed by extensive washing. This block was maximal, inasmuch as a higher concentration of toxin (3 μM) produced no additional inhibition (Fig. 1A). The average degree of inhibition was 46 ± 1% (n = 30) (Fig. 1B). We tested agents that influence other classes of Ca^{2+} channels to determine which might be responsible for the remaining transmission. Synaptic transmission was unaffected by FPL 64176, a powerful agonist of L-type channels (n = 4) (*13*), or nimodipine, a specific blocker of L-type channels (n = 6) (Fig. 1C). Likewise, application of 30 nM ω-Agatoxin IVA (ω-Aga-IVA), which potently blocks P-type Ca^{2+} channels (*14*), had no effect on transmission (Fig. 1D) (*15*). This was true regardless of whether ω-Aga-IVA was applied before (n = 14) or after (n = 2) ω-CTx-GVIA. The ω-CTx-GVIA– and ω-Aga-IVA–resistant transmission was completely and reversibly eliminated by removal of external Ca^{2+} ions (Fig. 1D). These experiments demonstrate that substantial excitatory synaptic transmission can be supported by a Ca^{2+} channel that is not of the N-, L-, or P-type.

The Q- and R-type Ca^{2+} channels in cerebellar granule neurons are resistant to blockade by ω-CTx-GVIA, nimodipine, and ω-Aga-IVA at concentrations sufficient to eliminate N-, L-, and P-type channels, respectively (*5, 6*). The Q- and R-type Ca^{2+} channels appear to be generated by α_{1A} and α_{1E} subunits (*3–5*). The Q-type channels are completely blocked by 1.5 μM ω-CTx-MVIIC and are largely suppressed by ω-Aga-IVA at 1 μM (*4, 6*), a concentration 100 to 1000 times that needed to block P-type channels (*14*). In contrast, R-type channels are little affected by either of these treatments (*5*). These characteristics enabled us to determine the contribution of Q- or R-type channels to hippocampal synaptic transmission. Application of 5 μM ω-CTx-MVIIC completely abolished the transmission that was not mediated by N-, L-, or P-type Ca^{2+} channels (n = 14) (Fig. 2A). Neuronal excitability was unaffected by toxin, as indi-

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