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23. The five contiguous nucleotides CAAGG that are invariant for all four FTZ-F1 binding sites in *ftz* (consensus $P_yCAAGGP_yCP_uCCP_u$) (*5*) make up the first half of the dyad, separated by three nucleotides from the second half-site, which is mismatched at two out of five positions. The FTZ-F1 binding sequence is shifted upstream of the GGTCRC motif that is recognized by many hormone receptors.
24. A random-primed, 0- to 16-hour embryo cDNA library in λgt11 was probed with a concatenated, synthetic FTZ-F1 DNA binding site (positions −267 to −299 of *ftz* terminated by two Sal I sites) labeled with ^{32}P by nick-translation. Colonies [8 × 10^6 plaque-forming units (pfu)] were screened at a density of 2 × 10^4 pfu per plate, as described [C. Vinson, K. L. LaMarco, P. F. Johnson, W. H. Landschulz, S. L. McKnight, *Genes Dev.* **2**, 801 (1988)], except that the duplicate filter was left on the plate for 10 hours and the binding buffer was supplemented with heat-denatured salmon sperm DNA (5 μg/ml). Plaque-purified clone 81 was plated and screened with ^{32}P-labeled wild-type and mutant FTZ-F1 binding sites. Synthesis of the recombinant protein was induced and the protein was extracted from a lysogen of clone 81 as described [M. Miyamoto *et al.*, *Cell* **54**, 903 (1988)], except that the lysis buffer was supplemented with leupeptin (5 μg/ml), pepstatin (5 μg/ml), and aprotinin (0.75 μg/ml). Electrophoretic mobility shift and methylation interference analyses were performed as described (*5*) except that the gel matrix was agarose (1%). For the methylation interference study, we used a 16- to 19-hour embryo nuclear extract (lanes 2 and 7) as a source of native FTZ-F1 protein.
25. The 1043–amino acid ORF was reconstructed from two overlapping cDNA clones isolated from a 0- to 4-hour embryo cDNA library [N. H. Brown and F. C. Kafatos, *J. Mol. Biol.* **203**, 425 (1988)] and a 2- to 4-hour embryo cDNA library (*9*), respectively. The first methionine in the ORF is presumed to be initiating. DNA sequencing was by the dideoxynucleotide method and data were assembled with the UWGCG sequence analysis programs [J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984)]. Like many developmental genes, *FTZ-F1* contains the *opa* repeat sequence $(CAG)_x$ [K. A. Wharton, B Yedvobnick, V. G. Finnerty, S. Artavanis-Tsakonas, *Cell* **40**, 55 (1985)], which is translated into stretches of Q (residues 39 to 47 and 361 to 367).
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Ca^{2+} Permeability of KA-AMPA–Gated Glutamate Receptor Channels Depends on Subunit Composition

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NMDA (*N*-methyl-D-aspartate) receptors and non-NMDA receptors represent the two major classes of ion channel–linked glutamate receptors. Unlike the NMDA receptor channels, non-NMDA receptor channels have usually been thought to conduct monovalent cations only. Non-NMDA receptor ion channels that can be gated by kainic acid (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) are formed by the glutamate receptor subunits GluR1, GluR2, and GluR3. These subunits were expressed in various combinations in *Xenopus* oocytes so that their permeability to divalent cations could be studied. At physiological resting potentials, KA and AMPA elicited inward calcium currents in oocytes expressing GluR1, GluR3, and GluR1 plus GluR3. In contrast, oocytes expressing GluR1 plus GluR2 or GluR3 plus GluR2 showed no such permeability. Thus, in neurons expressing certain KA-AMPA receptor subunits, glutamate may trigger calcium-dependent intracellular events by activating non-NMDA receptors.

THE GLUTAMATE-GATED ION CHANnels include those receptors activated by NMDA, KA, and AMPA (*1, 2*). The conductance mechanism of these ionotropic glutamate receptor (GluR) subtypes is cationic (*3*): NMDA receptor channels are permeable to monovalent cations and Ca^{2+} (*4–7*) whereas non-NMDA receptor channels have negligible permeability to divalent cations (*1, 8*). Many of the important physiological and pathological functions of NMDA receptors, for example in synaptic plasticity, learning, excitotoxic cell death, and neurological disorders (*9–11*), are, at least in part, consequences of the Ca^{2+} permeability of their integral ion channels. Although it is generally maintained that Ca^{2+} permeability of non-NMDA receptor channels is very low or absent (*4, 5, 12*), some KA-gated channels have been reported to be permeable to Ca^{2+} (*13, 14*), making this a controversial issue.

To test the permeability properties for divalent cations of the channels formed by different combinations of the KA-AMPA receptor subunits GluR1, GluR2, and GluR3 (*15, 17–19*), we expressed these subunits in various combinations in *Xenopus* oocytes that had been injected with cRNA (RNA transcribed in vitro from the cDNA clones). The ion channels were activated with KA, the most efficacious agonist at these receptors (*15, 17–19*), and ion permeability properties were analyzed under voltage clamp in test buffers of different ionic composition (*20*). In normal Ringer solution, the inward current through all combinations of GluR ion channels was carried mainly by an inward Na^+ flow because elimination of Na^+ from the external medium almost completely abolished the inward currents, whereas elimination of Cl^- neither changed the amplitudes nor shifted the reversal potentials (Fig. 1) (*21*). The outward currents seen at positive membrane potentials, which presumably indicate outward flow of K^+, remained unchanged when the external ions were exchanged. These findings indicate that, in normal Ringer, the inward current through GluR channels is carried mainly by Na^+ and K^+.

To test the GluR channels for calcium permeability, we used a Na^+,K^+-free medi-

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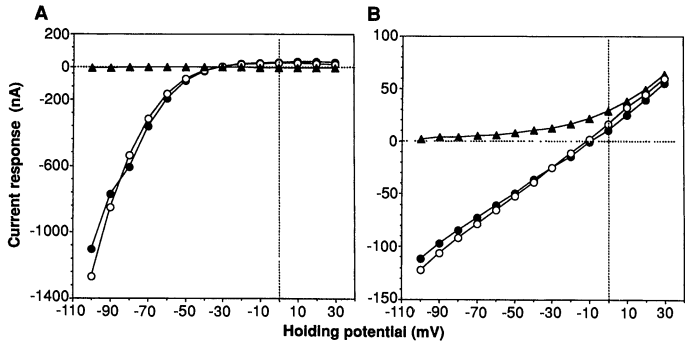

Fig. 1. Current responses to 100 μM KA as a function of membrane potential in normal Ringer (●), Cl^--free Ringer (○), and Na^+,K^+-free Ringer (▲) (*20*). (**A**) Data obtained from oocytes injected with GluR1 cRNA (2 ng) and (**B**) GluR1 plus GluR2 cRNA 1:10 (0.2 ng plus 2 ng). Voltages were stepped by 10 mV between −100 mV and +30 mV; data are averages from three oocytes.

um supplemented with 10 mM Ca^{2+} (Ca^{2+}-Ringer) (*20*). For this experiment, the following predictions could be made: if the channels were impermeable for Ca^{2+}, no inward currents would be elicited, whereas if the channels had Ca^{2+} permeability, some inward current would be observed. Any influx of Ca^{2+} into the oocyte, however, has been shown to trigger Ca^{2+}-activated Cl^- channels (Cl_{Ca} channels), which exist endogenously in the oocyte membrane (*22–24*). This would lead to an outward flux of Cl^- that would contribute to the total inward current. Although this Cl^- flux would increase the observed signal, it would complicate quantification of the fraction of the inward current actually carried by Ca^{2+}. We found that GluR1, GluR3, and GluR1 plus GluR3 exhibited a significant inward current, whereas when GluR2 was coexpressed with either GluR1 (1:1) (*25*) or GluR3 (10:1) (*25*) this inward current was eliminated (Fig. 2). The GluR2 subunit alone was not analyzed because of its weak current responses in oocytes (*17, 19*). The two splicing variants of GluR2, $GluR2_{flop}$ and $GluR2_{flip}$ (*26*), behaved similarly. Currents could be elicited by KA, domoic acid, quisqualic acid, and AMPA, but not by NMDA. Responses to 100 μM KA were completely and reversibly blocked by 100 μM 6,7-dinitroquinoxaline-2,3-dione (CNQX), a selective blocker of non-NMDA receptors (*27*). The median effective concentration (EC_{50}) for KA (*15*) was not changed in Ca^{2+}-Ringer solution. The current-voltage (*I-V*) curves of GluR1, GluR3, and GluR1 plus GluR3 in Ca^{2+}-Ringer (Fig. 3, A and B) were similar to the strongly inwardly rectifying curves seen in normal Ringer solution (*17*). However, *I-V* curves of GluR1 plus GluR2 and GluR2 plus GluR3 (10:1) (*25*) were different in Ca^{2+}-Ringer in that they exhibited inward current only at extremely negative (more negative than −100 mV) holding potentials (Fig. 3, A and B), instead of the linear *I-V* relations observed in normal Ringer (*17*). The inward currents seen with GluR1, GluR3, and GluR1 plus GluR3 in a medium that contained Ca^{2+} as the only permeable cation suggested that these receptor channels are permeable to Ca^{2+}. Because the reversal potentials of currents carried by Ca^{2+} should shift on variation of the Ca^{2+} concentration, we tested GluR1 channels for such a shift in a low-Na^+ buffer and in a Na^+-free buffer. In both buffers we found a depolarizing shift (Fig. 3C), suggesting that Ca^{2+} carries the observed current. As expected, the Na^+-free

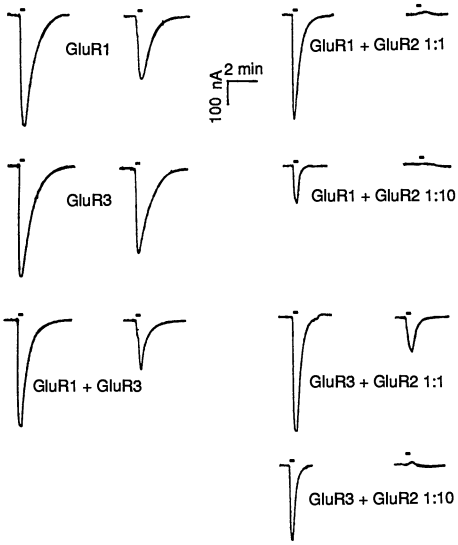

Fig. 2. Comparison of current responses in normal Ringer solution and Ca^{2+}-Ringer (*20*) to pulse application (25 s, indicated by bars) of 100 μM KA. Oocytes were injected with different single GluR subunit cRNAs (2 ng) or combinations of cRNAs (2 ng plus 2 ng for 1:1 combinations; 0.2 ng plus 2 ng for 1:10 combinations), as indicated. For each pair of traces, the one on the left was recorded in normal Ringer solution and the one on the right was recorded from the same oocyte in Ca^{2+}-Ringer. Oocytes injected with different combinations of cRNAs are from different experiments, so current amplitudes cannot be compared directly (but see Fig. 3, A and B, for direct comparison).

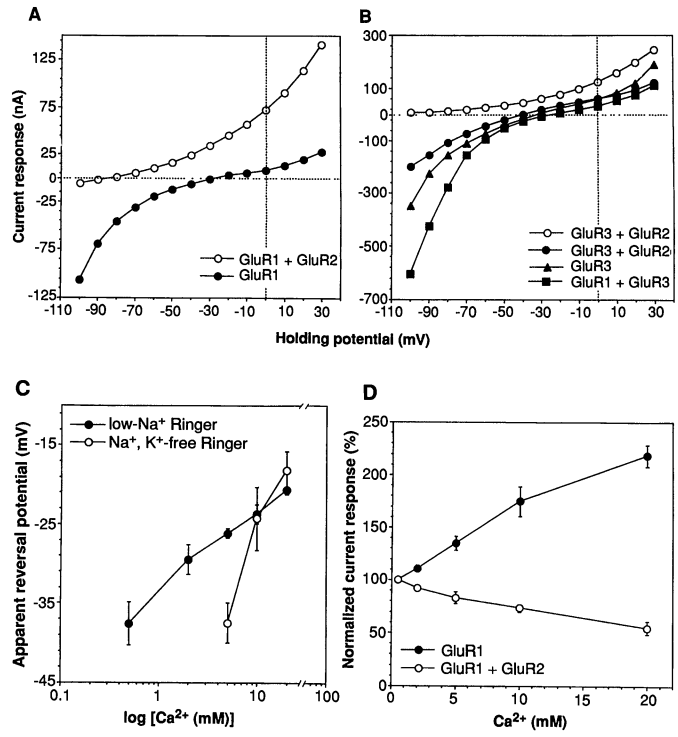

Fig. 3. (**A** and **B**) Current responses to 100 μM KA as a function of membrane potential in Ca^{2+}-Ringer (*20*). (A) Data obtained from oocytes injected with GluR1 cRNA (●) (2 ng) or GluR1 plus GluR2 cRNA 1:10 (○) (0.2 ng plus 2 ng) and (B) GluR3 cRNA (▲) (2 ng), GluR3 plus GluR2 cRNA 1:1 (●) (2 ng each), GluR3 plus GluR2 cRNA 1:10 (○) (0.2 ng plus 2 ng), and GluR1 plus GluR3 cRNA 1:1 (■) (2 ng each). Recordings were performed as in Fig. 1; data are averages from three to six oocytes. (**C**) Effect of Ca^{2+} concentration on the reversal potential of GluR1 current responses to 100 μM KA in low-Na^+ Ringer (●) and Na^+,K^+-free Ringer (○) (*20*). Oocytes were tested 4 days after injection with GluR1 (2 ng). Data are averages ± SEM from two to three oocytes. (**D**) Comparison between GluR1 and GluR1 plus GluR2 for Ca^{2+} dependence of amplitude of current responses to 100 μM KA. Oocytes were injected with GluR1 cRNA (●) (2 ng) or GluR1 plus GluR2 cRNA 1:10 (○) (0.2 ng plus 2 ng) and tested in low-Na^+ Ringer 4 days after injection. Low-Na^+ Ringer was used instead of Na^+, K^+-free Ringer so that inward currents from GluR1 plus GluR2 could be recorded. Data represent averages ± SEM from five to seven oocytes and were normalized to amplitudes measured in 0.5 mM Ca^{2+} to facilitate comparison.

buffer caused the steeper shift. Also, GluR1 responses in low-Na^+ buffer increased linearly with the Ca^{2+} concentration whereas GluR1 plus GluR2 responses decreased (Fig. 3D), suggesting that Ca^{2+}, rather than carrying currents through GluR1 plus GluR2 channels, either inhibits the receptor or interferes with the permeation of monovalent cations.

Several other divalent cations (Mg^{2+}, Sr^{2+}, and Ba^{2+}) were tested for their permeability properties. GluR1 plus GluR2 channels, as expected, were found to be impermeable to these cations, whereas the cations were able to elicit substantial inward currents at GluR1 channels. Even Mg^{2+}, which blocks NMDA receptor channels, is capable of penetrating GluR1 channels. The apparent reversal potentials of these divalent cations were very similar, suggesting that the GluR1 channel has only a low selectivity. For three oocytes we determined the following apparent reversal potentials: Ca^{2+}, -22.0 ± 3.4 mV; Mg^{2+}, -31.3 ± 1.7 mV; Sr^{2+}, -28.3 ± 1.4 mV; and Ba^{2+}, -19.2 ± 0.95 mV. Thus, the relative permeability sequence is $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$. However, because the contribution by Cl_{Ca} currents to the KA responses in Ca^{2+}-Ringer is unknown, it is impossible to estimate a meaningful reversal potential. Thus, the position of Ca^{2+} in this permeability sequence of divalent cations must be considered tentative. Currents seen with Mg^{2+}, Sr^{2+}, and Ba^{2+} were generally smaller than those observed with Ca^{2+}, probably because Sr^{2+} and Ba^{2+} are only poor activators of Cl_{Ca} channels (*22, 23*) and Mg^{2+} is entirely inactive (*24*).

In conclusion, our data suggest that the Ca^{2+} permeability of KA-AMPA receptor channels varies with the subunit composition of the receptor. GluR1 plus GluR2 and GluR2 plus GluR3 apparently form Ca^{2+}-impermeable heterooligomeric channels, whereas GluR1, GluR3, and GluR1 plus GluR3 channels allow the influx of Ca^{2+}. The secondary activation by Ca^{2+} of Cl_{Ca} channels endogenous to the oocyte, although precluding an estimation of the size of the Ca^{2+} current through KA-AMPA channels, by itself is evidence that enough Ca^{2+} permeates some KA-AMPA receptor channels to trigger a biological response in the oocyte. Secondary activation of Cl_{Ca} channels in oocytes has been used with other receptor systems as an indicator for Ca^{2+} flux through ion channels, for example the *Torpedo* acetylcholine receptor channel (*28*) and the NMDA receptor channel (*29*).

Because GluR receptor subunits are differentially expressed (*17, 18, 30*), different cell types may express different subunit combinations that are either Ca^{2+}-permeable or Ca^{2+}-impermeable. This could explain the discrepancies found in the literature regarding the Ca^{2+} permeability of non-NMDA receptors. Iino and co-workers described two types of KA-evoked responses in cultured hippocampal neurons (*14*), one of which, type II, bears resemblance to GluR1, GluR3, and GluR1 plus GluR3 responses. The type II response is strongly rectifying, and it is also observed in Ca^{2+}-Ringer. The KA type I response, on the other hand, resembles GluR1 plus GluR2 and GluR3 plus GluR2 responses. It has a linear *I-V* relation and is not seen in Ca^{2+}-Ringer solutions. Iino and co-workers (*14*) estimated that the Ca^{2+} permeability of the type II channels was about 13-fold higher than that of the type I channels and only 2.7-fold lower than Ca^{2+} permeability of NMDA receptor channels in the same cells.

Ca^{2+} influx into neurons through NMDA receptor channels is believed to cause excitotoxic cell damage (*10*). However, blocking NMDA channels does not always prevent this damage, an observation that can be explained by our data, which predict that Ca^{2+} flux through KA-AMPA receptor channels may underlie some aspects of excitotoxicity. Additionally, Ca^{2+} flux through KA-AMPA receptor channels may contribute to a variety of Ca^{2+}-dependent plastic processes.

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16. The glutamate receptor originally called GluR-K1 (*15*) was shown to be activated by both KA and AMPA (*17–19*). Thus, the existence of separate KA and AMPA receptors is uncertain, and we therefore refer to GluR-K1 as a KA-AMPA receptor. We also changed the original name GluR-K1 to the pharmacologically neutral name GluR1 (*17*).
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20. Standard Ringer solution for amphibia (normal Ringer): 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes, pH adjusted to 7.2 with NaOH. Cl^--free Ringer: normal Ringer, but NaOH and KOH instead of NaCl and KCl, pH adjusted to 7.2 with methanesulfonic acid. Na^+,K^+-free Ringer: normal Ringer, but NaCl and KCl isoosmotically substituted with *N*-methylglucamine, (NMG), pH adjusted to 7.2 with HCl. Ca^{2+}-Ringer: Na^+,K^+-free Ringer, but with 10 mM $CaCl_2$. Low-Na^+ Ringer: 75 mM NaCl, 2.5 mM KCl, 0.5, 2, 5, 10, or 20 mM $CaCl_2$, NMG for osmotic balance, 10 mM Hepes, pH adjusted to 7.2 with HCl. We prepared cRNA transcripts and performed oocyte injections as described (*15*). Recordings were made 4 to 10 days after cRNA injection under two-electrode voltage clamp at a holding potential of -70 mV (except for *I-V* curves) with a DAGAN 8500 amplifier. Voltage electrodes had a resistance of 4 to 8 megohms and were filled with 3 M KCl; current electrodes had a resistance of ~1 megohm and were filled with 0.25 M CsCl, 0.25 M CsF, 100 mM EGTA, pH 7.2. All drugs were pulse-applied (1.6 ml) by superfusion (4 ml/min) in a 300-μl chamber; peak currents were recorded.
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25. The current amplitude and the shape of the *I-V* curve of GluR3 plus GluR2 (and, to a much lesser extent, that of GluR1 plus GluR2) in Ca^{2+}-Ringer strongly depended on the relative proportion of the two cRNAs that were injected. If GluR3 and GluR2 cRNAs were injected at a molar ratio of 1:1, some inward current and a slightly inwardly rectifying *I-V* curve were observed (Fig. 3B). However, when GluR2 was injected in tenfold molar excess, no inward current was seen and the *I-V* curve was outwardly rectifying (Fig. 3B). This is interpreted as follows: when the two cRNAs are injected at a 1:1 ratio, homooligomeric channels and heterooligomeric channels are formed, with both types of channels contributing some current; with GluR2 in excess, formation of homooligomeric GluR3 channels presumably is reduced, and the response is largely due to the heterooligomeric channels. Because GluR2 by itself gives only very weak responses in oocytes (*17, 19*), its tenfold excess (which presumably results in a large number of homooligomeric GluR2 channels) does not contribute significantly to the current measured and thus does not obscure the analysis of heterooligomeric channel properties. In contrast to the subunit combinations involving GluR2, the GluR1 and GluR3 channels do not seem to interact in the oocyte membrane, at least not in such a way as to form heterooligomeric channels with permeability properties different from those of the respective homooligomeric channels.
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