Aromatase Enzyme Activity and Sex Determination in Chickens

ALEX ELBRECHT* AND ROY G. SMITH

During development, the genotype of the zygote determines the nature of the gonad, which then determines the male or female phenotype. The molecular events underlying this process are just beginning to be defined. A single treatment of chicken embryos with an aromatase inhibitor (which blocks the synthesis of estrogen from testosterone) at a stage when their gonads were bipotential caused genetic females to develop a permanent male phenotype. These sex-reversed females developed bilateral testes that were capable of complete spermatogenesis and had the physical appearance and behavior of normal males. This result identifies aromatase as a key developmental switch in the sex determination of chickens.

SEXUAL DIFFERENTIATION IS A SEquential, ordered process. The genotype of the zygote determines the nature of the gonads, which leads to the development of either a male or female phenotype. In mammals, a gene for the putative testes-determining factor (TDF) has been identified on the Y chromosome (*1, 2*). This candidate TDF is related to the DNA-binding proteins HMG1 and HMG2 and to the product of the yeast gene *Mc*, a possible transcription factor. This suggests a role for TDF as the master switch for other genes downstream in the pathway of sexual differentiation. Whether a TDF homolog exists in birds remains to be determined.

Likely downstream targets for TDF include anti-Müllerian hormone (AMH) and the gonadal steroids. AMH causes regression of the embryonic Müllerian ducts and may act as a morphogen for testes development (*3, 4*). In addition, AMH may also regulate gonadal steroid hormone levels by inhibiting the enzyme aromatase (*5*). This enzyme catalyzes the final step in the biosynthesis of the gonadal steroids, that is, the conversion of androgens to estrogens. The gonadal steroids may participate in gonadal morphogenesis itself and are necessary for the development of male and female secondary sex characteristics. The ratio of estradiol to testosterone may also play a crucial role in sex determination because estrogen is known to attenuate the effects of AMH (*3*).

We used an aromatase inhibitor to evaluate the role of the gonadal steroids, specifically the conversion of testosterone to estradiol, in gonadal differentiation of the early chicken embryo. We treated embryos with 1 mg of the aromatase inhibitor (±)5-(*p*-cyanophenyl)-5,6,7,8-tetrahydroimidazo[1,5-α]pyridine hydrochloride (AI) on day 5 of egg incubation to test whether aromatase inhibition is crucial in controlling gonadal differentiation (*6, 7*). This caused essentially 100% (Table 1) of all hatchlings to vent sex as males (*8*). Vent sexing exploits the dimorphism in the cloaca to determine phenotypic sex shortly after hatching (*9*). Doses of 0.01 mg were not effective (Table 1); doses ≥0.1 mg resulted in virtually all hatchlings developing a male phenotype (Table 1), but higher doses reduced hatching. Treatment was effective during a developmental window extending from preincubation (day 0) to day 7 of incubation (Table 1). The timing of treatment for successful sex reversal of females is consistent with the evidence that, before day 7 of incubation, chick embryonic gonads are bipotential (*3*). This was confirmed in several hundred embryos treated with AI.

To monitor more easily changes in development of chickens as a function of age and genetic sex, an autosexing cross was used. Autosexing chickens are obtained by crossing different breeds possessing sex-linked feather color mutations. Males and females are identified at hatching by their unique feather colors. Rhode Island Red males, *b+ b+*, were crossed with Plymouth Barred Rock females, *B/w*, (Avian Services, Frenchtown, New Jersey) to obtain offspring in which females have predominantly black feathers and males have barred feathers. A 1-mg dose of AI on day 5 caused three of four (*10*) treated females to develop a complete male adult phenotype with testes (Fig. 1). One of the three was killed after 24 weeks, and the remaining two (#1977 and #1981) were killed after 31 weeks. For each animal, blood was taken to determine the concentration of gonadal steroids in the serum; the gonads were removed and fixed for histology in Bouin's fixative. Unlike control females, the treated females exhibited low serum estradiol concentrations and high serum testosterone concentrations (Table 2), red nonfatty livers, a lack of serum turbidity from circulating lipids, and an absence of abdominal fat.

In the normal female, the right gonad and oviduct begin to regress during embryogenesis and at maturity females have a single ovary and oviduct (Fig. 2A). In contrast, mature females treated with AI had two gonads without oviducts. The gonads clearly resembled testes, although they appeared somewhat smaller and more irregular than the testes of a normal male of the same age (Fig. 2B).

Testes from control males (Fig. 2, C and D) and treated females (Fig. 2, E to H) at 31 weeks of age contained seminiferous tubules and interstitial cells. Spermatogenesis is characterized by the presence of normal Sertoli cells, spermatogonia, primary and secondary spermatocytes, round spermatids, and elongated spermatids. It is of particular significance that seminiferous tubules were present in both the left and right gonads of treated females (Fig. 2, E to H). In some areas of the tubules, spermatocyte and spermatid nuclei had apparently undergone division without cytoplasmic cleavage; however, multinucleated cells can occur normally in chicken seminiferous epithelium (*11*). In some areas abnormal condensation of the spermatid nuclei was associated with

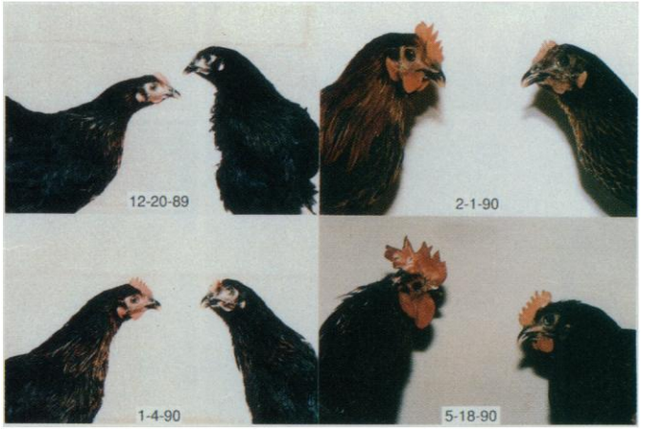

Fig. 1. Chronology of sex-reversed chicken. In each panel the control chicken is on the right and the treated chicken is on the left. Both are genetic females identified by feather color and DNA dot blots (*26*). The numbers in the inset boxes refer to the date the animals were photographed (month-day-year). Both were hatched on 10-11-89. Note the masculine hackle, the large comb, and wattles of the treated chicken.

Department of Animal Biochemistry and Molecular Biology, Merck Sharp and Dohme Research Laboratories, Rahway, NJ 07065.

*To whom correspondence should be addressed.

the appearance of large vacuoles. Two of the gonads also had a small cyst-like structure. Nevertheless, the histological appearance of the testes of sex-reversed females was similar in size and germ cellular associations to that in the normal male testis. The stages within an individual tubule from a genetic female frequently exhibited more heterogeneity in the stage of spermatogenesis within an individual cross section than in the seminiferous epithelium from the normal male. Normal males treated with AI also exhibited normal spermatogenesis, and the cell associations in the seminiferous epithelium were not distinguishable from those of nontreated males.

Two other aromatase inhibitors were tested for their ability to cause phenotypic sex reversal. The steroidal inhibitor 1,4,6-androstatriene-3,17-dione (*12*) prevented regression of the right gonad in chicken embryos when administered at a dose of 2 mg per egg. Similarly, the nonsteroidal inhibitor 6-[(4-chlorophenyl)(1*H*-1,2,4-triazol-1-yl)methyl]-1-methyl-1*H*-benzotriazole (*13*) was effective at a dose of 1 mg per egg. Administration of neither testosterone (0.01 mg or 0.1 mg) nor tamoxifen (0.5 mg) caused reversal of the female sexual phenotype. This is probably because testosterone would be converted to estradiol by aromatase, and tamoxifen would not increase androgen levels, which is probably necessary for phenotype conversion. Thus, the results are specific for treatment with an aromatase inhibitor and are not observed upon treatment with other steroids or antiestrogens. Moreover, AI did not bind to chicken oviduct estrogen or androgen receptors (*14*); its effects on gonadal differentiation are not due to antiestrogen or androgen-like activities.

If the effects of AI are due to its inhibition of estradiol synthesis, they should be inhibited by coadministration of estradiol. To monitor both sexual phenotype and genetic sex, we used vent sexing and feather sexing, respectively (Table 3). Coadministration of estradiol inhibited the effects of the aromatase inhibitor, and the chicks vent sexed as females upon hatching. Estrogens not only prevented the masculinizing effect of AI but also caused feminization of males. Aromatase therefore plays a critical role on gonadal and hence sexual differentiation.

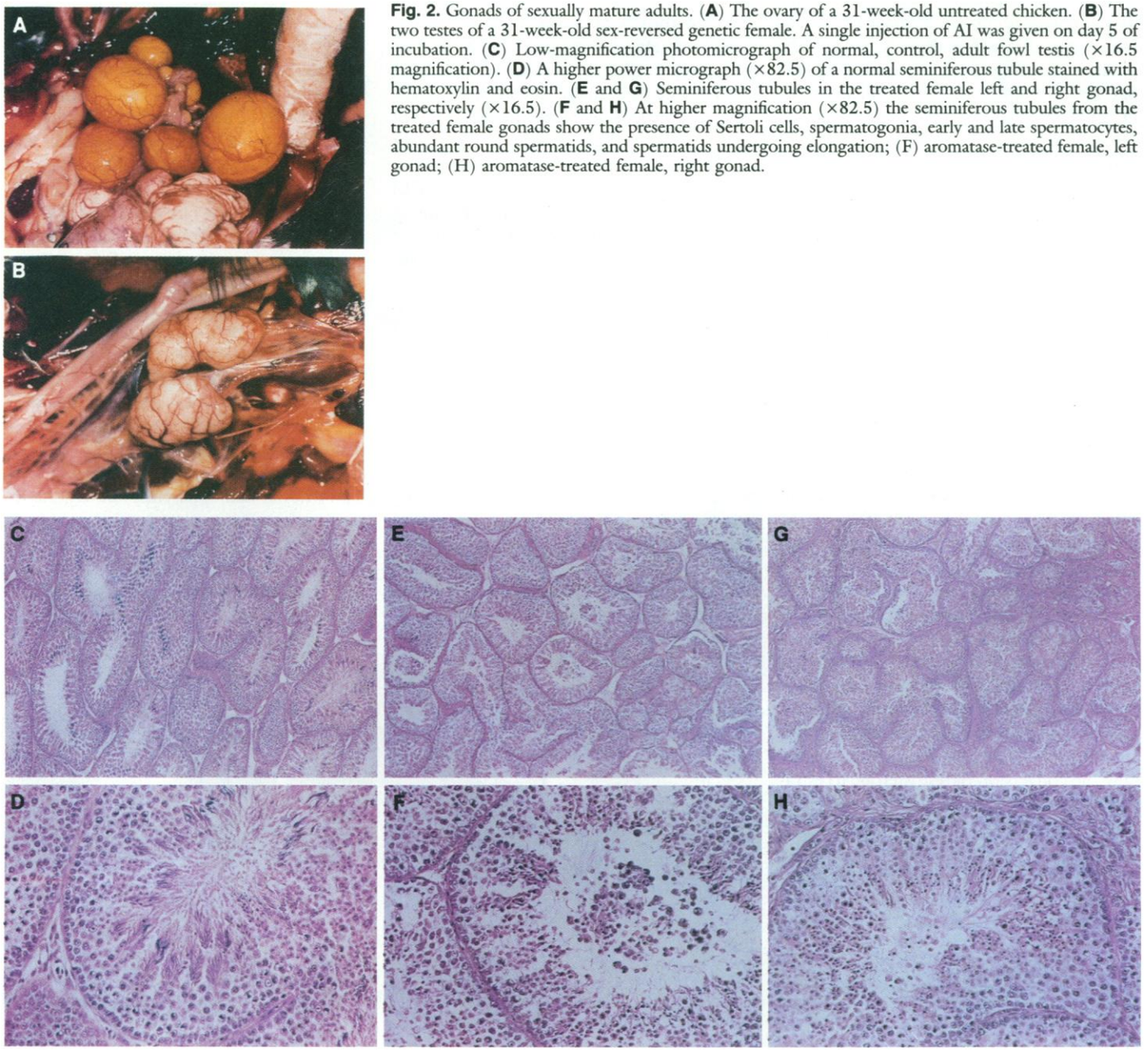

Fig. 2. Gonads of sexually mature adults. (**A**) The ovary of a 31-week-old untreated chicken. (**B**) The two testes of a 31-week-old sex-reversed genetic female. A single injection of AI was given on day 5 of incubation. (**C**) Low-magnification photomicrograph of normal, control, adult fowl testis (×16.5 magnification). (**D**) A higher power micrograph (×82.5) of a normal seminiferous tubule stained with hematoxylin and eosin. (**E** and **G**) Seminiferous tubules in the treated female left and right gonad, respectively (×16.5). (**F** and **H**) At higher magnification (×82.5) the seminiferous tubules from the treated female gonads show the presence of Sertoli cells, spermatogonia, early and late spermatocytes, abundant round spermatids, and spermatids undergoing elongation; (F) aromatase-treated female, left gonad; (H) aromatase-treated female, right gonad.

Table 1. Effect of AI on vent sex. Peterson X Arbor Acre eggs (Avian Services, Frenchtown, New Jersey) were injected once as indicated with various doses of AI dissolved in 0.1 ml of saline. Injections were made into the egg white under the air sac with a 1-cm^3 syringe attached to a 23-gauge, 1-inch needle. The holes were sealed with Scotch tape. Phenotypic sex was determined at hatching by an experienced vent sexer. Genetic sex was determined by examination of feather length and structure. A and B are two separate experiments. M, male; F, female.

Day	Treatment	M	F	Method
	Experiment A			
0	Uninjected	46	39	Vent
		46	39	Feather
0	Saline	21	23	Vent
		21	23	Feather
0	0.01 mg of AI	27	20	Vent
		21	26	Feather
0	0.1 mg of AI	56	0	Vent
		32	24	Feather
5	0.05 mg of AI	59	5	Vent
		31	33	Feather
5	0.1 mg of AI	55	3	Vent
		26	32	Feather
5	Saline	33	36	Vent
		30	39	Feather
5	Uninjected	34	42	Vent
		28	48	Feather
7	0.1 mg of AI	45	2	Vent
9	0.1 mg of AI	44	21	Vent
		29	36	Feather
	Experiment B			
5	0.1 mg of AI	52	0	Vent
5	0.5 mg of AI	36	0	Vent
5	1.0 mg of AI	46	0	Vent
5	Saline	26	30	Vent

Table 2. Gonadal steroid hormone levels in serum. Estradiol and testosterone were determined in serum samples with commercial radioimmunoassay (RIA) kits; 1978 is a treated, genetic male that displayed no obvious changes because of treatment; 1977 and 1981 are genetic females but phenotypic males.

Chicken	Estradiol (ng/dl)	Testosterone (ng/dl)
Control female	129	*
Control male	2	219
1977	12	224
1978	5	412
1981	2	109

*The testosterone concentration could not be determined accurately because of high lipid levels in the serum.

Table 3. Reversal of the effects of AI by estrogen. Peterson X Arbor Acre eggs were injected on day 5 of incubation with 0.1 mg of AI, 0.1 mg of 17-β-estradiol(E_2), or both, dissolved in 0.05 ml of 1,2-propane-diol. Chicks were vent-sexed and feather-sexed 1 day after hatching. Data are expressed as number of chicks in each group.

Treatment	Vent sex		Feather sex	
	Male	Female	Male	Female
Vehicle	36	35	35	36
AI	45	0	16	29
AI + E_2	3	50	26	27
E_2	0	51	27	24
Control	24	16	24	16

To demonstrate that AI inhibits chicken aromatase, as well as that of humans and rats (*6*), we showed that AI inhibited chicken aromatase activity when expressed from a transfected cDNA clone (*15*) in CV-1 cells and in microsomes from hen ovaries (*16*). Second, the fact that estrogen could reverse the effects of AI (Table 3) indicates that AI is acting by inhibiting estradiol synthesis.

Our results demonstrate that gonadal steroid hormones are involved in sex determination in chickens. A single treatment with three different aromatase inhibitors at a critical period of embryonic development conferred a male phenotype on genetically female chickens. Not only did the animals have the secondary sex characteristics and behavior of males, but they possessed two testes, and the testes produced sperm (*17*).

Inhibition of embryonic aromatase activity results in increases in testosterone at the expense of decreased local estradiol concentrations, causing the development of testes and therefore of male sex determination. An endogenous aromatase inhibitor that controls gonadal differentiation remains to be identified, but AMH (*5*) and 5β-androstan-17β-ol-3-one (*18*) are both candidates. Although 100% of the treated females had developed a male phenotype upon hatching, only about 50% continued to grow as males and developed mature testes. The rate of development of male secondary sexual characteristics also appeared to be retarded; the testes were irregularly shaped and sperm counts were low. The reason for these deficiencies is unknown and might be corrected by coadministration of avian AMH and AI. AMH has been shown to play a role in male gonadal development and phenotype in mammals (*4*). Furthermore, AMH is secreted earlier in embryonic development and at higher concentrations in males than in females (*3, 19*). The pleiotropic effects of AMH on male development including regression of Müllerian ducts, morphogenesis of the testes, and inhibition of aromatase enzyme activity would result in male sex determination. Because early embryonic gonads are capable of synthesizing sex steroids (*20*), the delayed expression and lower concentrations of AMH in female embryos permit aromatization of the gonadal steroids and formation of estrogens. The estrogens in the developing left ovary protect against the effects of AMH once it is secreted (*21*). The factors involved in regulating the expression of AMH are not known.

Regulation of aromatase may participate in gonadal differentiation in other species. For example, in both rabbit (*22*) and human (*23*) embryonic gonads, the capacity for the ovary to produce estrogen corresponds to the time of onset of testosterone synthesis in the testis, which occurs before the histological differentiation of the ovary (*24*). Pseudohermaphroditism of a human female fetus has been associated with a placental aromatase deficiency (*25*). These reports together with the results described herein implicate the regulation of aromatase activity as a crucial and general event in the control of gonadal differentiation and sex determination.

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probed with the plasmid pUDG0603 [H. Kodama *et al.*, *Chromosoma* **96**, 18 (1987)]. This plasmid contains a member of a repetitive element that is unique to the W chromosome. The presence of the W chromosome in the sex-reversed chickens identifies them as genetic females.

27. We thank S. Mizuno for providing plasmid pUDG0603, M. J. McPhaul, and J. D. Wilson for the chicken aromatase cDNA clone, and G. M. Mark and S. Zavodny for expressing the aromatase cDNA. In addition, we thank E. Mills for help with the chickens, M. Harper for preparation of the manuscript, and D. Lamb for assistance in interpreting the histology of the gonads.

5 June 1991; accepted 15 October 1991

High Probability Opening of NMDA Receptor Channels by L-Glutamate

CRAIG E. JAHR

Synaptic plasticity can be triggered by calcium flux into neurons through synaptically activated *N*-methyl-D-aspartate (NMDA) receptor channels. The amplitude and time course of the resulting intracellular calcium transient depend on the number of open NMDA receptor channels and the kinetics of their activation. Short applications of L-glutamate to outside-out patches from hippocampal neurons in the presence and absence of MK-801 revealed that about 30 percent of L-glutamate–bound channels are open at the peak of the current. This high probability of opening suggests that very few channels are required to guarantee a large, localized postsynaptic calcium transient.

SYNAPTIC ACTIVATION OF NMDA receptor channels leads to a slowly rising, long-lasting excitatory postsynaptic current (EPSC) at a variety of excitatory synapses in the vertebrate central nervous system (*1–8*). The slow time course of the NMDA receptor EPSC is caused by a slow opening rate of NMDA channels after binding the transmitter L-glutamate and by repetitive opening resulting from slow unbinding (*7–9*). Although the kinetics of NMDA receptor channel openings in the continuous presence of agonists have been studied in detail (*10–12*), sudden jumps in agonist concentrations evoke desensitizing responses that cannot be easily predicted from steady-state recordings (*7–9, 13, 14*). These characteristics of channel gating are also difficult to address with the use of synaptic preparations because presynaptic release parameters are not readily controlled. In the experiments described here outside-out patches from hippocampal neurons were used as surrogate synapses to determine opening probability while controlling variables such as receptor saturation (*7–9, 15*). By using the anticonvulsant MK-801 (*16*), which blocks only open NMDA receptor channels [and in the time frame of these experiments, blocks them irreversibly (*17–19*)], the probability that a liganded channel will open and the open probability at the peak of the current can be estimated.

The superimposed records in Fig. 1A are averages of responses of an outside-out patch evoked by short, saturating applications of L-glutamate [1 mM for 8 ms (*7–9*)] in the presence of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (2 μM). The time course of these currents is the same as that of the NMDA receptor component of EPSCs recorded in cultured neurons (*7, 8*) and those in the hippocampal slice (*4–6*). If both the control solution and the L-glutamate solution were switched to solutions containing 20 μM MK-801, a single 8-ms application of L-glutamate resulted in a current attenuated in both amplitude and duration (Fig. 1B). Ten seconds after activating this current, the solutions were exchanged for those without MK-801 and the patch was washed with control solution for 30 s. Subsequent L-glutamate applications evoked currents that were reduced in amplitude relative to pre–MK-801 trials (Fig. 1C), but with similar time courses (Fig. 1D). Because high concentrations of MK-801 were used, most of the channels that were opened by this single pulse of L-glutamate should have been blocked by MK-801. Only the channels that were bound by agonist but never actually opened, or opened for a short time, would be available for subsequent activation. The percentage decrease in charge transfer produced by a single application of L-glutamate in the presence of MK-801 was 70 ± 3.6% (mean ± SE) in 20 μM MK-801 (n = 8), 65 ± 2.2% in 10 μM MK-801 (n = 7), and 46 ± 5.4% in 3 μM MK-801 (n = 5). The percent inhibition of charge transfer at the highest concentration provides an estimate of the probability that a channel bound by L-glutamate will open before L-glutamate unbinds ($P_{o(total)}$ = 0.7) (*20*). This estimate depends on the assumptions that MK-801 only blocks channels that are open and that the block is irreversible at negative holding potentials over this period of time (*17–19*). A plot of the charge transfer evoked by each application of L-glutamate versus time demonstrates these phenomena (Fig. 1E). When the patch was exposed to MK-801 but not in conjunction with L-glutamate, responses to L-glutamate after washing out MK-801 were not diminished (Fig. 1A). In nine trials (eight patches), applications of MK-801 alone had no effect on subsequent responses to L-glutamate (P = 0.44; paired t test). In addition, after a single L-glutamate application in the presence of MK-801, subsequent responses to L-glutamate remained stable, indicating that recovery from MK-801 blockade was not significant in these conditions (Fig. 1E).

If the probability of a bound channel

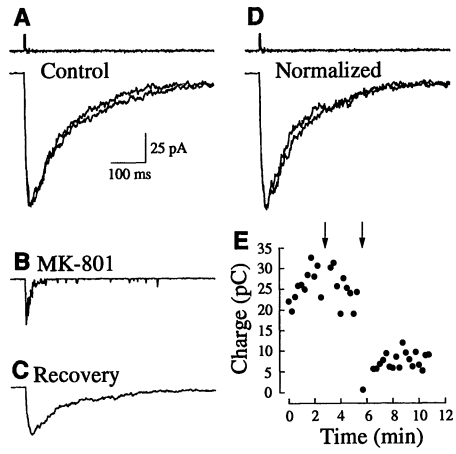

Fig. 1. L-glutamate activates NMDA receptor channels with a high probability. (**A**) Superimposed averages of currents evoked by 8-ms applications of 1 mM L-glutamate in an outside-out patch in the continued presence of 20 μM glycine. The two averages represent responses recorded before (average of 11 responses) and after exposure to 20 μM MK-801 (9 responses) in the absence of L-glutamate. (**B**) Single response of the same patch as in (A) to 1 mM L-glutamate in the continuous presence of MK-801 (20 μM). (**C**) Averaged response of the same patch to L-glutamate after superfusing the patch with control solution for 30 s. (**D**) Superimposition of averaged currents recorded before and after the single L-glutamate response in the presence of MK-801. The averaged current in (C) has been normalized to the peak amplitude of the response in (A). The similarity in time courses of the two averages suggests that channels with similar kinetic properties underlie both responses. (**E**) Charge transfer of individual responses to L-glutamate in the same patch before and after MK-801 treatment which did (right arrow) and did not (left arrow) coincide with an application of L-glutamate. Charge transfer was measured from records 1024 ms long. The top records in (A) and (D) are open-tip currents (*15*).

Vollum Institute L474, Oregon Health Sciences University, Portland, OR 97201.