natively, the presence of the ζ_2 homodimer might prevent the coupling of the TCR to phosphoinositide turnover in complexes containing ζ_2. It should be noted that a dichotomy was found in MA 5.8 between the ability of anti–CD3-ε and antigen to induce IL-2 production. A similar comparison must be made for tyrosine phosphorylation in the new variants to determine if CD3-ζη is important for coupling TCR occupancy to a tyrosine kinase pathway.

Although the generation of IP and the activation of PKC-dependent phosphorylation correlate with the amounts of CD3-ζη, this does not constitute proof that these events are directly related to TCR composition. However, the correlation between the structural and the physiological data does suggest a novel biological mechanism whereby a single ligand-binding molecule can initiate independent intracellular pathways due to an association with different signal transducing elements.

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6. Two (B10.A × AKR)F_1 mice were injected subcutaneously with 2B4.11 T cells and treated with intraperitoneal injections of anti–CD3-ε (2 μg per mouse every other day for 14 days). The tumors that appeared at the site of inoculation in both animals were excised and the cells cultured. One tumor line expressed low levels of TCR, as judged by immunofluorescence staining with MAb A2B4-2 (anti–TCR-α). Cloning by limiting dilution yielded five subclones, all of which stained only weakly with anti–TCR-α and responded poorly (with IL-2 production or inhibition of growth) to stimulation with antigen or anti–CD3-ε. Immunoblots detected little or no CD3-ζ chain on the five clones. The other tumor line was heterogeneous in its expression of anti–TCR-α–reactive material and was cloned twice, yielding 6 and 150 subclones, respectively. Immunoblots with anti–CD3-ζ revealed many of these subclones to be deficient in CD3-ζη (2 of 6 in the first subcloning, 28 of 80 tested in the second subcloning). Of these, two T cell hybridomas from independent subclonings that expressed the lowest levels of CD3-ζη were selected for further analysis: EV.3 and 2M.44.
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17. In experiments (n = 2) to analyze tyrosine phosphorylation of CD3-ζ, unlabeled 2B4.11 and EV.3 cells were stimulated with LK 35.2 cells and a 4% final concentration of anti–CD3-ε supernatant. Antigen receptors were immunopurified as above and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose, and CD3-ζ chains phosphorylated on tyrosine residues were detected with affinity-purified antibodies to phosphotyrosine and ^{125}I-labeled protein A (*14*). To normalize for differences in receptor number between the two cell types, 2B4.11 and EV.3 cells were labeled with ^{32}P and stimulated with doses of PMA that give maximal and stoichiometric CD3-γ phosphorylation (*15*). The level of phosphorylation was determined by densitometry after NEPHGE/SDS-PAGE. Relative tyrosine phosphorylation of CD3-ζ is expressed as the ratio of arbitrary absorbance units: (EV.3ζ/EV.3γ) to (2B4.11ζ/2B4.11γ). In experiments (n = 2) to analyze phosphoinositide hydrolysis, relative receptor density was evaluated by indirect immunofluoresence. Comparison of this technique with densitometric analysis of PMA-induced phosphorylation yielded comparable results when used for normalization. The data represent the arithmetic average of two independent experiments each.
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Limited Immunological Recognition of Critical Malaria Vaccine Candidate Antigens

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Current vaccine development strategies for malaria depend on widespread immunological responsiveness to candidate antigens such as the zygote surface antigens and the sporozoite coat protein, the circumsporozoite (CS) protein. Since immunological responsiveness is controlled mainly by genes mapping within the major histocompatibility complex (MHC), the humoral immune response to the zygote surface antigens and the cytotoxic T lymphocyte (CTL) response to the CS protein were examined in MHC-disparate congenic mouse strains. Only two of six strains responded to the 230-kilodalton zygote surface antigen and another two strains responded to the 48/45-kilodalton surface antigen. From two mouse strains, expressing between them five different class I MHC molecules, there was recognition of only a single CTL epitope from the CS protein, which was from a polymorphic segment of the molecule. The restricted CTL response to this protein parallels the restricted antibody response to this protein observed in humans and mice. These findings suggest that subunit malaria vaccines now being developed may be ineffective.

THE ABILITY OF AN INDIVIDUAL TO respond to a given antigen is governed by immune response (*Ir*) genes and the repertoire of immunoreactive lymphocytes. *Ir* genes, which encode the polymorphic MHC molecules, function by the ability of their gene products to bind T cell antigenic sites derived from the antigen (*1*). *Ir* genes control both CTLs (by class I MHC molecules) and helper T cells. Helper T cells are responsible for helping antibody production by B cells as well as for antibody-independent cellular immunity (by class II MHC molecules). Although there are constraints on which regions of a protein can function as T cell epitopes (*2*), it was expected that most foreign proteins would contain sufficient T epitopes such that they would be recognized by most members of the species. Indeed, foreign proteins such as hepatitis B surface antigen with a monomeric size of 26 kD and staphylococcal nuclease (17 kD) are recognized by most strains of congenic mice that differ in their MHC-encoding region (H-2) (*3*).

Natural immunity to malaria is often slow to develop, and in regions endemic for malaria, children are fully susceptible for a number of years. Most adults in endemic regions are parasitemic because of poor im-

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munity to blood stage parasites and little or no immunity to sporozoite or hepatic stage parasites (*4*). Despite repeated infections, most adults from malaria-endemic regions lack transmission blocking immunity that would block infection of malaria to mosquitoes and thus prevent further transmission of the disease (*5, 6*). If such lack of immunity reflects a lack of responsiveness to critical individual antigens as a result of *Ir* gene control, the subunit vaccine approach for malaria would be challenged. We examined whether there was widespread *Ir* gene–controlled nonresponsiveness to critical antigens.

We initially examined the humoral immune response to zygote surface antigens that are known targets of antibody-dependent transmission blocking immunity (*7*). Different strains of H-2 congenic mice were immunized with three injections of purified zygotes (*8*) of the 3D7 clone of *Plasmodium falciparum* (*9*). The sera produced were used to immunoprecipitate either ^{125}I surface-labeled zygotes or [^{35}S]cysteine metabolically labeled zygotes (*10*). Three major surface antigens are targets for transmission-blocking antibodies (*11–13*). The 230-kD and 48/45-kD antigens are present in the gametocytes that circulate within the human host and are readily iodinated, whereas the 25-kD antigen is expressed on the zygote and ookinete, both of which occur only in the mosquito (*14*). As shown in Fig. 1a, most strains were nonresponsive to either the 230-kD or 48/45-kD antigens. Although there was some variation within strains, most of the variation was between strains. Some of the weaker immunoprecipitation of the 230-kD antigen (for example, sera 1 and 5 from the B10.D2 group) may represent coprecipitation with the 48/45-kD antigen, as described (*15*). Note that only one strain, B10.BR, recognized a 40-kD gamete surface antigen. The antibody response to the 25-kD antigen, apparent from the experiment depicted in Fig. 1a, was shown better when the antigen was metabolically labeled with [^{35}S]cysteine (Fig. 1b). In contrast to the immune responses to the 230-kD and 48/45-kD antigens, most strains responded well to this antigen.

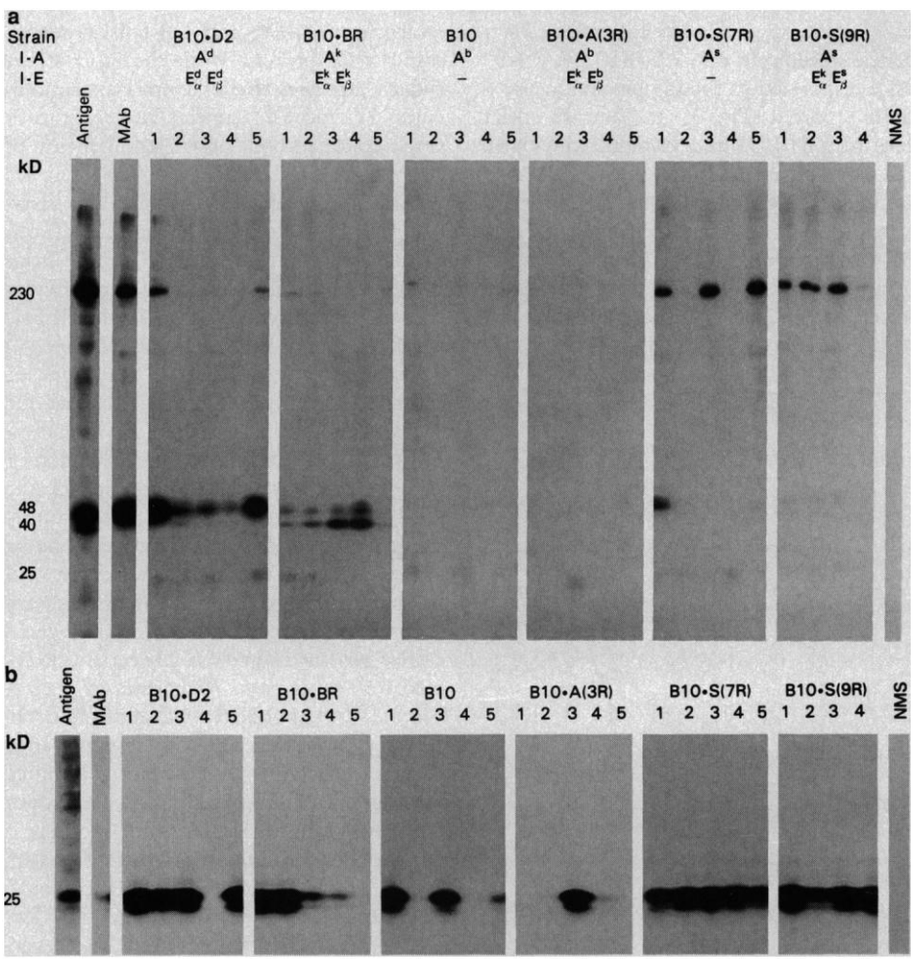

Fig. 1. (**a**) Restricted recognition of 230-kD and 48/45-kD gamete/zygote surface antigens by MHC-disparate congenic mouse strains. Immunoprecipitation of ^{125}I surface-labeled zygotes by sera from individual mice, as indicated (*10*). Lanes: MAb, immunoprecipitation by a mixture of monoclonal antibodies (MAbs) IIC5-B10 (*12*) and 1B3 (*11*) to the 48/45-kD and the 230-kD antigens, respectively; antigen, a portion of nonprecipitated antigen was separated by electrophoresis; NMS, a pool of preimmune normal sera. I-A and I-E refer to the two class II molecules expressed by these different H-2 congenic strains. B10 and B10.S(7R) strains do not express an I-E molecule. (**b**) Widespread recognition of 25-kD zygote surface antigen. Immunoprecipitation of [^{35}S]cysteine metabolically labeled zygotes by sera as in (a). Lane MAb, immunoprecipitation by MAb 1C7 (*26*).

Although the CD4 (T helper) epitopes have not been determined for the 230-kD and 48/45-kD gamete surface antigen vaccine candidates, our data suggest that they must be limited in number. Since antibody is the critical form of immunity (*7*), an infection with a parasite not containing a helper T epitope for a given antigen would not stimulate an antibody response to that antigen (*16*). Although gametocytes infectious to mosquitoes appear only 12 or more days after blood infection, infection would not boost immunity, and transmission to mosquitoes would go unimpeded. We propose that these parasite antigens have evolved to have limited T cell epitopes so as not to boost transmission blocking immunity during infection. In contrast, antibody response to the 25-kD protein is not restricted. This protein is expressed in the mosquito and probably not in humans (*14*). Thus, there may never have been immune pressure on this protein to evolve limited T cell epitopes, as antibody to 25-kD antigen is not induced during infection (*6*).

The different *Ir* gene control of the antibody immune response to the different gamete surface antigens indicates that T cells that recognize one membrane-associated antigen cannot provide help for B cells that recognize a different antigen. This is in contrast to the immune response to certain viral proteins, where helper T cells specific for an internal viral protein can provide help for B cells specific for a surface viral protein (*17*).

We next examined the immune response to the circumsporozoite (CS) protein, which is a target antigen of antibody before hepatocyte invasion by sporozoites and of CTLs, probably after invasion of hepatocytes (*18, 19*). The antibody response to the CS protein of *P. falciparum* is restricted in terms of the number of individuals that respond to it in both humans (*20*) and mice (*22*). The studies in mice showed that this was the result of *Ir* gene control. Furthermore, whereas proliferating T cells from 40% of humans living in a malaria-endemic region of West Africa are unable to respond to any of a series of overlapping peptides that span the entire protein, in the 60% who do respond, most of the response is specific for the polymorphic segment of the protein (*20*). While antibody to CS protein can protect from malaria sporozoites, murine

studies suggest that CTLs can protect more efficiently (*18, 19*). In terms of the CS protein–specific CTL response, human epitopes have not been determined, but CTLs from B10.BR (H-2^k) mice immunized with either *P. falciparum* sporozoites or a CS-recombinant vaccinia virus recognize the CS protein (*18*). When the epitope was mapped by the use of CTLs derived from CS-vaccinia–immunized mice the response was shown to be restricted to a single peptide epitope from a polymorphic segment of the protein (*18*). This epitope was also recognized by CTLs derived from sporozoite-immunized mice.

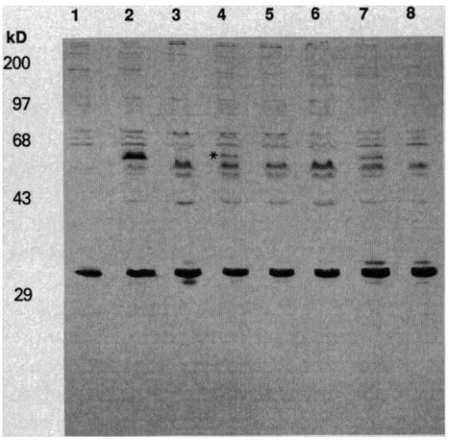

Fig. 2. Expression of CS protein by transfected fibroblasts (*18*). Immunoblot of electrophoresed solubilized extracts from CS-vaccinia–infected 3T3 cells; (lane 2); CS-transfected 3T3 cells (lane 3 with clone A2, lane 4 with clone A3, lane 5 with clone A5, and lane 6 with clone B1) and uninfected untransfected 3T3 (H-2^d) fibroblasts (lane 1), and CS-transfected (lane 7) and -untransfected (lane 8) L cell (H-2^k) fibroblasts with peroxidase-conjugated MAb to CS protein (*21*). (*) Expression of the 60-kD CS protein in both CS-vaccinia–infected and CS-transfected fibroblasts. The observed size of the CS protein after gel electrophoresis is greater than the predicted size (*27*).

The degree of *Ir* gene–controlled responsiveness to the CS protein was determined by transfecting fibroblasts (3T3 cells) of a different MHC haplotype (H-2^d) with the CS gene (*18*). Expression was confirmed by immunoblot (*21*) (Fig. 2) and immunofluorescence (data not shown). B10.BR (H-2^k) and B10.D2 (H-2^d) mice were then immunized intravenously with 10^7 PFU (plaque-forming units) of the CS (7G8 clone)–recombinant vaccinia virus (*22*). Spleen cells taken after 3 weeks were stimulated with either CS-transfected fibroblasts of the same H-2 haplotype, or CS-vaccinia–infected syngeneic spleen cells (*18, 23*). B10.BR (H-2^k) mice responded to the CS protein as previously reported (Fig. 3). In contrast, spleen cells from B10.D2 mice immunized with the CS-recombinant vaccinia virus could not be stimulated to generate specific CTL for CS-transfected 3T3 cells, although they generated vaccinia-specific CTLs (Fig. 3). Similar results were obtained for BALB/c (H-2^d) mice. It is unlikely that these findings were due to failure of antigen processing by the 3T3 cells, since these cells can process a similarly transfected protein of the human immunodeficiency virus (*23*), as well as vaccinia-specific proteins (Fig. 3). Also, spleen cells taken from 7G8 sporozoite-immunized B10.D2 mice were stimulated with CS-transfected 3T3 cells. These also failed to generate CTLs even though the donor mice developed specific antibodies to CS protein of equivalent titer to those produced by B10.BR mice whose spleen cells were capable of generating CTLs after sporozoite inoculation. As a further control, addition of interleukin-2 (IL-2)–containing supernatant to the cultures did not permit the generation of CS-specific CTLs from CS-vaccinia–immunized or sporozoite-immunized B10.D2 mice. Thus the defect in response was due to lack of ability of the protein to stimulate CTLs, not helper T cells.

Thus, for two strains of MHC-disparate mice, one (which expresses three class I molecules: K^d, D^d, and L^d) fails to recognize the CS protein, while the other strain (which expresses two different class I molecules: K^k and D^k) appears to recognize a single epitope from a polymorphic region of the protein. The limitation of T cell epitopes within the CS protein, as well as the variation within the single known CTL T cell epitope, may be the result of escape from immune surveillance of infected hepatocytes containing parasites lacking CS T cell epitopes or bearing variant T cell epitopes.

Our results highlight the challenges of subunit vaccine development (at least for sporozoite and sexual stages). The limitation of the CTL response as well as limitation of and variation within CS protein–specific CD4 (T helper) epitopes (*20, 24*), strongly suggest that the CS protein either alone or as expressed by a recombinant vector, will not be a suitable vaccine. It may be necessary to fuse the CS protein with an immunogenic carrier protein and rely solely on antibody-mediated protection. Recipients of such a vaccine would not always benefit from natural boosting of their antibody response after natural challenge, since boosting requires recognition of CS-specific T cell epitopes (*16*). For transmission-blocking vaccines it will probably be necessary to fuse nonmalarial proteins as a source of stimulation for T helper cells, and again natural boosting during infection will often not occur. However, a "cocktail" vaccine containing all zygote surface antigens could be expected to have greater efficacy. It is possible that humans may have a less restricted response to these critical immunogens than mice. However, it should be noted that two major murine T helper epitopes from the CS protein coincide precisely with the two immunodomin-

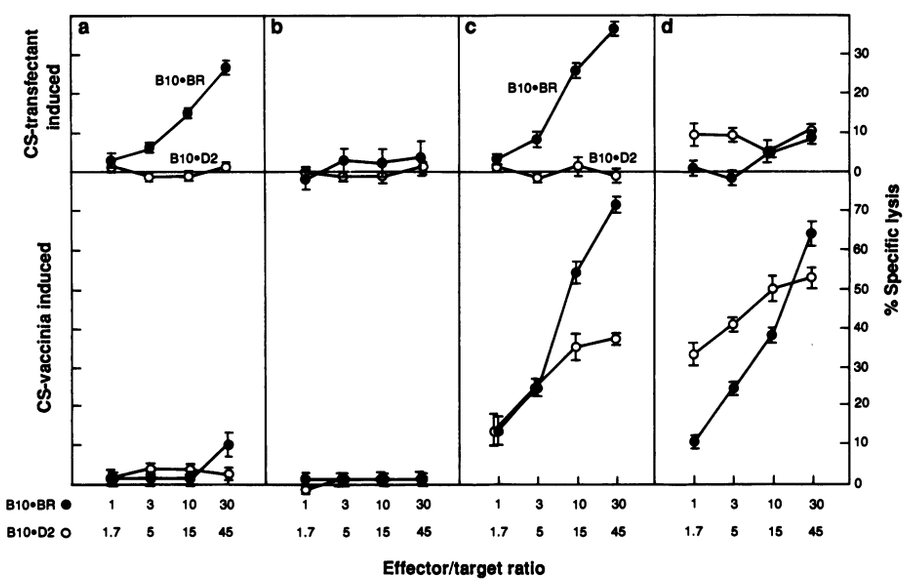

Fig. 3. The CS protein is a target for CTL in H-2^k mice, but not H-2^d mice. ●, Spleen cells pooled from two CS-vaccinia–immunized B10.BR mice were stimulated in vitro with CS-transfected L cells (clone 1) or CS-vaccinia–infected spleen cells (*18*), and CTL activity was measured against a panel of modified or unmodified target L cells as indicated. These CTLs do not recognize L cells transfected with an irrelevant gene (*18*). ○, spleen cells pooled from two CS-vaccinia–immunized B10.D2 mice were stimulated in vitro with either CS-vaccinia–infected spleen cells or with CS-transfected 3T3 cells (*18*). The transfectant used in the experiment here was clone A3, but similar results were obtained with clones A2, A5, and B1. Specific lysis was calculated as described (*18*). Error bars represent ±SEM (triplicate assays).

ant human T cell epitopes from this protein (*20, 22, 25*). The challenge facing us now is to use the skills of modern molecular immunology to identify means of circumventing immunological nonresponsiveness.

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8. Mature *P. falciparum* gametocytes [3D7 clone (*9*)] were produced in vitro as described (*11*). Gametes were then allowed to exflagellate at room temperature (*11*) and purified by using a Nycodenz gradient [A. N. Vermeulen *et al.*, *J. Exp. Med.* **162**, 1460 (1985)]. Gametes were then further incubated for 5 hours (28°C, 80% humidity) to allow zygote formation. Mice were immunized with a primary intraperitoneal injection of 5×10^6 zygotes emulsified in complete Freund's adjuvant, and two subsequent injections of 5×10^6 zygotes emulsified in incomplete Freund's adjuvant at 21 days and 31 days after the primary injection. Mice were bled before immunization and at day 41, and sera were prepared.
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21. Cells were solubilized and denatured in RIP buffer (100 m*M* tris, *p*H 8.0, 150 m*M* NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 2 m*M* EGTA, 0.1 m*M* phenylmethylsulfonyl fluoride) in a volume of 0.2 ml. A 0.025-ml aliquot was then added to sample buffer [19 m*M* tris, 1.25% SDS, 192 m*M* glycine, 320 m*M* 2-mercaptoethanol, 10% (v/v) glycerol] and boiled at 100°C for 3 min. Samples were separated by SDS–polyacrylamide gel electrophoresis, and the gel was electroblotted overnight in transfer buffer (25 m*M* tris and 192 m*M* glycine in 15% methanol) onto a nitrocellulose membrane (0.2-μm pore size). Nitrocellulose blots were blocked with 0.3% Tween-20 in phosphate-buffered saline (PBS), washed in 0.05% Tween-20/PBS, and incubated for 2 hours with a 1:1000 dilution of peroxidase-conjugated monoclonal antibody to CS protein, 2A10 [F. Zavala *et al.*, *Science* **228**, 1436 (1985)] (Kirkegaard & Perry). Blots were then washed and reacted with 2.8 m*M* 4-chloro-1-napthol and 0.015% hydrogen peroxide in PBS. Reaction was stopped with distilled water.
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Single Subunits of the $GABA_A$ Receptor Form Ion Channels with Properties of the Native Receptor

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The α and β subunits of the γ-aminobutyric $acid_A$ ($GABA_A$) receptor were expressed individually in *Xenopus* oocytes by injection of RNA synthesized from their cloned DNAs. GABA-sensitive chloride channels were detected several days after injection with any one of three different α RNAs (α_1, α_2, and α_3) or with β RNA. The channels induced by each of the α-subunit RNAs were indistinguishable, they had multiple conductance levels (10, 19, 28, and 42 picosiemens), and their activity was potentiated by pentobarbital and inhibited by picrotoxin. The β channels usually expressed poorly but showed similar single channel conductance levels (10, 18, 27, and 40 picosiemens), potentiation by pentobarbital and inhibition by picrotoxin. The finding that both α and β subunits, examined separately, form GABA-sensitive ion channels with permeation properties and regulatory sites characteristic of the native receptor suggests that the amino acid sequences that confer these properties are within the homologous domains shared by the subunits.

GABA, A MAJOR INHIBITORY NEUrotransmitter, binds to specific receptors in the vertebrate central nervous system. Ligand binding to the $GABA_A$ receptor activates chloride channels of multiple conductance states (*1, 2*) that are modulated by barbiturates and the convulsant picrotoxin (*3*). Immunoprecipitation and purification studies indicate that the native $GABA_A$ receptor consists of at least two subunits (α and β) (*4–6*). Photoaffinity labeling with the GABA agonist, [^3H]muscimol, suggests that the ligand-binding site is on the β subunit (*7, 8*). Recently, cDNAs encoding one β and three α subtypes (α_1, α_2, and α_3) have been isolated from bovine brain (*9, 10*); their high degree of homology (~35% sequence identity) suggests that α and β subunits arose from gene duplication. We report that the α and β subunits of the $GABA_A$ receptor, expressed separately, are each able to form ligand-sensitive ion channels with normal conductance, ion selectivity, and pharmacological properties.

RNA was synthesized separately from α and β cDNAs and injected singly into *Xenopus* oocytes. Expression was assayed with two-electrode voltage-clamp and patch-clamp techniques (*11*). We detected GABA-evoked currents in oocytes injected with either α or β RNA (Fig. 1). The responses were dose-dependent, enhanced by 50 μ*M* pentobarbital, and reduced by 2.5 μ*M* picrotoxin. No response was seen to glycine (up to 100 μ*M*). Desensitization increased with elevation of GABA from 1 μ*M* to 1 m*M* (Fig. 1A). Measurements made during

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