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An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation

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THE phenomenon of long-term potentiation (LTP), a long lasting increase in the strength of synaptic transmission which is due to brief, repetitive activation of excitatory afferent fibres, is one of the most striking examples of synaptic plasticity in the mammalian brain. In the CA1 region of the hippocampus, the induction of LTP requires activation of NMDA (*N*-methyl-D-aspartate) receptors by synaptically released glutamate[1] with concomitant postsynaptic membrane depolarization[2–5]. This relieves the voltage-dependent magnesium block of the NMDA-receptor ion channel[6,7], allowing calcium to flow into the dendritic spine[8–10]. Although calcium has been shown to be a necessary trigger for LTP (refs 11, 12), little is known about the immediate biochemical processes that are activated by calcium and are responsible for LTP. The most attractive candidates have been calcium/calmodulin-dependent protein kinase II (CaM-KII) (refs 13–16), protein kinase C (refs 17–19), and the calcium-dependent protease, calpain[20]. Extracellular application of protein kinase inhibitors to the hippocampal slice preparation blocks the induction of LTP (refs 21–23) but it is unclear whether this is due to a pre- and/or postsynaptic action. We have found that intracellular injection into CA1 pyramidal cells of the protein kinase inhibitor H-7, or of the calmodulin antagonist calmidazolium, blocks LTP. Furthermore, LTP is blocked by the injection of synthetic peptides that are potent calmodulin antagonists and inhibit CaM-KII auto- and substrate phosphorylation. These findings demonstrate that in the postsynaptic cell both activation of calmodulin and kinase activity are required for the generation of LTP, and focus further attention on the potential role of CaM-KII in LTP.

Figure 1*a* shows a comparison between the tetanus-induced potentiation of the intracellular excitatory postsynaptic potential (e.p.s.p.) in cells recorded with electrodes filled with normal control recording solution, and cells recorded with electrodes which contained in addition 20 mM H-7, a potent but non-specific kinase inhibitor[24] (see Table 1). Control cells showed robust potentiation at 60 min, whereas H-7 filled cells showed a decremental potentiation which returned to baseline after ~30–40 min. The time course of this potentiation is similar to that seen following extracellular application of H-7 (ref. 21). Figure 1*b* shows that the magnitude of the LTP induced in the cells surrounding those which were impaled, as measured by monitoring field e.p.s.ps, was similar in the two populations of hippocampal slices.

Both CaM-KII and protein kinase C (PKC) have been proposed to play a role in LTP. A number of calmodulin (CaM) antagonists have been reported to block LTP, following bath application, to the entire hippocampal slice[22,25–27]. As was the case with H-7, however, it is unclear whether these compounds acted pre- and/or postsynaptically. To test whether activation of calmodulin in the postsynaptic cell is required for LTP, we recorded from cells with electrodes filled with 0.5 mM calmidazolium. Calmidazolium blocked LTP (Fig. 1*c*) in a manner similar to that seen when cells were filled with H-7. This could not be attributed to the effects of dimethyl sulphoxide (DMSO), the solvent for calmidazolium, as the control cells recorded with electrodes filled with 1% DMSO exhibited LTP. Figure 1*d* shows that the magnitude and time course of the LTP induced in the two populations of slices were again very similar. This set of experiments suggests that activation of CaM within the postsynaptic cell is required for the generation of LTP.

Calmidazolium, however, like other calmodulin antagonists tested on LTP, has actions in addition to its ability to antagonize calmodulin[28,29]. Furthermore, both H-7 and calmidazolium could slowly cross neuronal membranes, making it difficult to rule out absolutely effects on adjacent presynaptic terminals. It was therefore important to examine further the role of postsynaptic calmodulin in LTP using inhibitors of calmodulin that are more specific and less membrane-permeable. Recently a synthetic CaM-binding peptide (CBP), the sequence of which is based on the CaM-binding domain of CaM-KII, has been shown to bind potently to CaM and inhibit the CaM-dependent activation of CaM-KII (Table 1)[30]. In an initial set of experiments, we compared the effects on LTP of intracellularly applied CBP and CTP_2, a control peptide that shares sequence homology with CBP and has a similar isoelectric point, but is not a CaM antagonist (Table 1). For all experiments involving intracellular application of peptides, the same sample of peptide used for electrophysiological experiments was also tested biochemically, as described in Table 1. Figure 2*a* shows that LTP was blocked in cells recorded with electrodes containing CBP, whereas CTP_2 had no apparent effect on LTP. The simultaneously recorded field e.p.s.ps are plotted in Fig. 2*b* and demonstrate that there was no observable difference in the LTP induced in the two populations of hippocampal slices.

If the three N-terminal amino acids are removed from CBP, a peptide is obtained (CBP_{-3}) which has the same CaM antagonistic activity but, unlike CBP, does not block Ca^{2+}/CaM-independent substrate phosphorylation by previously autophosphorylated CaM-KII (Table 1)[30]. Thus, if CBP_{-3} differed from

TABLE 1 Inhibition of substrate phosphorylation

	CaM-KII Ca^{2+}/CaM-dependent IC_{50} (μm)	CaM-KII Ca^{2+}/CaM-independent IC_{50} (μm)	PKC IC_{50} (μm)
CBP	0.090	2.0	>150*
CBP_{-3}	0.090	>150*	>150*
CTP_2	170	>2,000*	—
H-7	20	32	8

The substrates for the two forms of CaM-KII were synapsin I (4–50 μM per assay) or the synthetic peptide MHRQETVDG-amide (single-letter amino-acid code; 15 μM); for PKC the substrate was syntide (15 μM) (ref. 30). IC_{50} values were determined as described previously[30]. The Ca/CaM-independent form of CaM-KII was prepared by autophosphorylation as described[30]. PKC was purified and assayed as described[38], except that chromatography on protamine-agarose was omitted. Final ATP concentration in all assays was 15 μM. The sequence of CBP is MHRQETVDCLKKFNARRKLKGAILTTMLA, that of CBP_{-3} is QETVDCLKKFNARRKLKGAILTTMLA, and that of CTP_2 is ILTTMLATRNFSGGK. CTP_2 was a gift from Ruthann Masarachia.
* No inhibition of kinase activity was observed at this concentration.

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CBP in its ability to block LTP, it would strongly suggest that the blockade of LTP by CBP requires the inhibition of CaM-independent CaM-KII activity in addition to its CaM antagonist activity. Figure 2*c* and *d* demonstrates that the blockade of LTP by CBP_{-3} is indistinguishable from the blockade by CBP. This result indicates that the ability of the two peptides to block LTP must be attributed to the inhibition of CaM. Taken together, the results using electrodes filled with calmidazolium, CBP, CBP_{-3} or CTP_2 indicate that the activation of postsynaptic CaM is a critical requirement for the generation of LTP. In addition, our results clearly demonstrate the power of using inhibitory peptides, synthesized from primary sequence data, to study synaptic physiology and intracellular biochemistry in mammalian brain tissue.

To test the possibility that the peptides may have exerted their effect by a non-specific depressant effect on synaptic transmission we examined the effects of CBP on LTP evoked in one pathway while simultaneously recording e.p.s.ps in response to an independent control pathway. Figure 3*a* shows that CBP blocked LTP in the intracellularly recorded cells, but had essentially no effect on the control e.p.s.ps. The field e.p.s.ps recorded in response to the two pathways are plotted in Fig. 3*b* which shows that LTP was induced in the tetanized pathway, whereas synaptic transmission in the control pathway was stable. Thus CBP has no observable effects on normal synaptic transmission.

The biochemical mechanisms underlying LTP in the CA1 region of the hippocampus have generated much research and discussion. The data presented here indicate that activation of postsynaptic CaM, presumably by the rise in dendritic spine Ca^{2+} initiated by tetanic stimulation, is a requisite step in the induction of LTP. Furthermore, we have demonstrated that postsynaptic kinase activity is also required for LTP. The simplest explanation for these findings is that LTP requires CaM-dependent activation of CaM-KII. The potential involvement of other kinases or CaM-dependent processes cannot be ruled out. The fact that CBP and CBP_{-3} do not inhibit PKC activity *in vitro* (Table 1), however, suggests that PKC alone cannot be responsible for LTP.

CaM-KII is a particularly favourable candidate to play a critical role in LTP because it makes up 20–40% of the protein

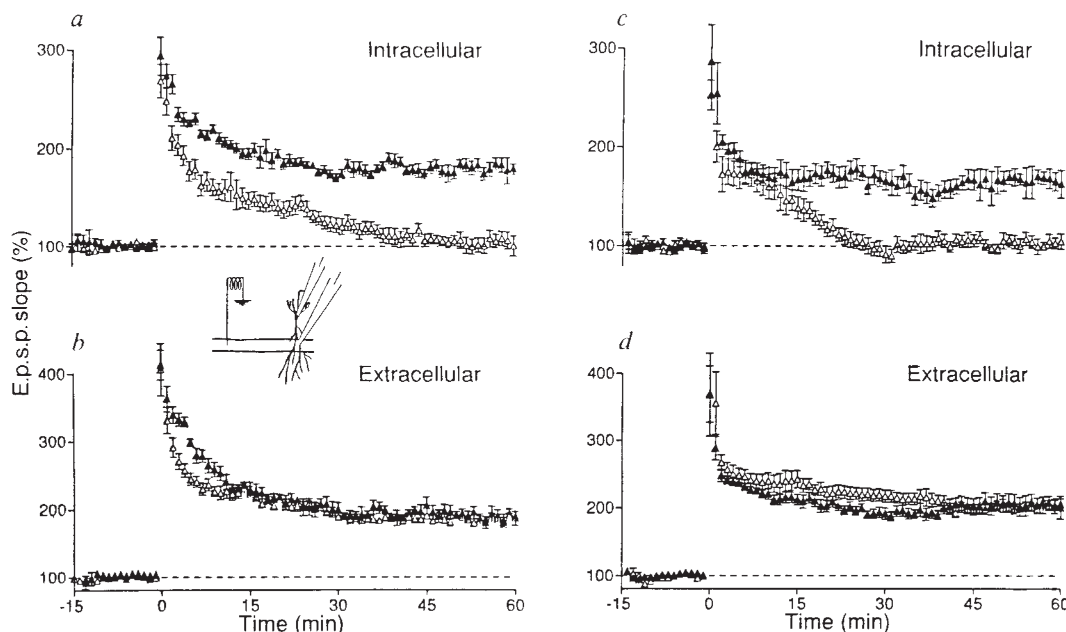

FIG. 1 Intracellular application of H-7, a protein kinase inhibitor, or calmidazolium, a CaM antagonist, blocks LTP. *a*, Comparison of changes in the magnitude of the initial slope of populations of cells recorded with microelectrodes containing either (△) H-7 (20 mM in 3 M CsCl; $n=8$) or (▲) CsCl (3 M; $n=6$) following LTP-inducing stimuli. Inset, a diagram of the position of the stimulating and recording microelectrodes. *b*, Magnitude and time course of LTP induced in the two populations of slices as measured by changes in the initial slope of simultaneously recorded field e.p.s.ps. For this and all subsequent figures, symbols represent the mean ± s.e.m. *c*, Comparison of the e.p.s.p. initial slope following LTP-inducing tetani in populations of cell recorded with microelectrodes containing either (△) calmidazolium (0.5 mM in 1% DMSO; $n=5$) or (▲) 1% DMSO alone ($n=5$). *d*, The initial slope of the field e.p.s.ps is plotted and demonstrates that the magnitude of LTP induced in the two populations of slices was the same.

METHODS. Hippocampal slices were prepared as previously described[39]. All experiments involved stimulation of the Schaffer collateral/commissural afferents at 0.1 Hz using a bipolar, stainless steel stimulating microelectrode placed in stratum radiatum. The medium in all experiments was (mM); NaCl (119), KCl (4), $MgSO_4$ (4), $CaCl_2$ (4), NaH_2PO_4 (1), $NaHCO_3$ (26), dextrose (11), picrotoxin (0.1). A surgical cut was made between CA1 and CA3 regions to prevent epileptiform discharges. Intracellular recordings were made in stratum pyramdale using microelectrodes (40–70 MΩ) filled with 3 M CsCl. To monitor the occurrence and magnitude of LTP in each hippocampal slice, field e.p.s.ps were simultaneously recorded in stratum radiatum using a low resistance (2–6 MΩ) microelectrode containing 3 M NaCl. During the course of each experiment, a cell was impaled and after a 10–20 min waiting period, a 10–20 min stable baseline was obtained. Tetanic stimulation consisted of two 1 s tetani at 100 Hz separated by 20 s and was given at 1.5 times the control stimulation strength. Only those experiments in which the field e.p.s.p. initial slope remained greater than 120% of its control value at 60 min after the tetanus, were included in the data analysis (~85% of experiments). At all times during the experiment, except during the tetani, hyperpolarizing current (d.c.) was passed so that the membrane potentials of the cells were kept at between −70 and −90 mV. This routinely prevented the generation of Ca^{2+} spikes or other voltage-dependent events which might result in the pairing of membrane depolarization with synaptic stimulation, the minimal requirements for the induction of LTP. To ensure that during the tetani adequate depolarization was achieved to induce LTP, the hyperpolarizing holding current was removed. All data were digitized on line using an IBM AT-compatible microcomputer. The maximum initial slope of each e.p.s.p. was calculated using a modified version of pClamp 4 (Axon Instruments). Each slope measurement in an individual experiment was normalized to the average value of all points on the baseline (at least 10 min before LTP induction). Individual experiments were then aligned with respect to the time of tetanic stimulation and divided into 60s bins, each of which was averaged to give the graphs illustrated. H-7 was obtained from Seikagaku and calmidazolium from Sigma. Peptides were synthesised and analysed as described[30]. The concentration of these compounds within the recorded cells is unknown. Hyperpolarizing current (d.c.) would be expected to retard the diffusion of these basic molecules into the cell, in part accounting for the requirement of concentrations within the recording microelectrode that are ~1,000-fold higher than the respective IC_{50} values.

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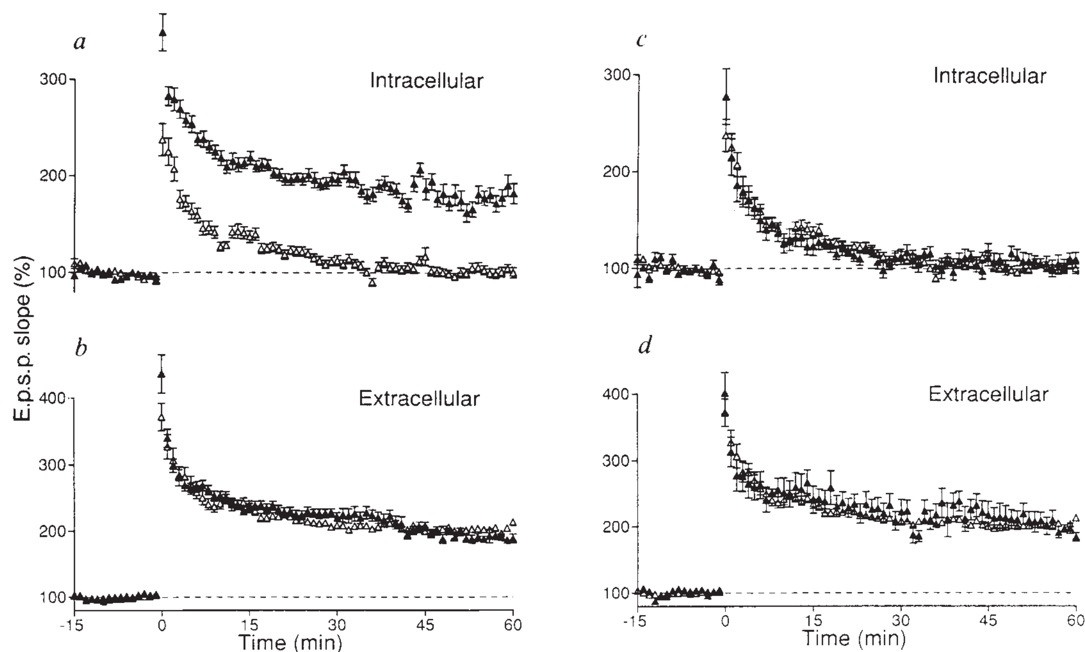

FIG. 2 Effect of intracellular application of the CaM-binding peptide (CBP) on LTP. *a*, The magnitude of the initial e.p.s.p. slope in populations of cells recorded with microelectrodes containing either (△) CBP (190 μM: $n=11$) or (▲) the control peptide CTP_2 (190 μM; $n=8$). *b*, The initial field e.p.s.p. slope recorded in the two populations of slices demonstrating that the LTP was essentially identical in the two populations. *c*, The peptide, CBP_{-3}, which inhibits CaM activity but does not block Ca/CaM-independent CaM-KII substrate phosphorylation, has the same ability to block LTP as does CBP. Microelectrodes contained 190 μM CBP_{-3} ($n=6$). The data for the CBP-filled cells are the same as shown in *a*. *d*, LTP generated in the field potential is very similar for the two populations of slices.

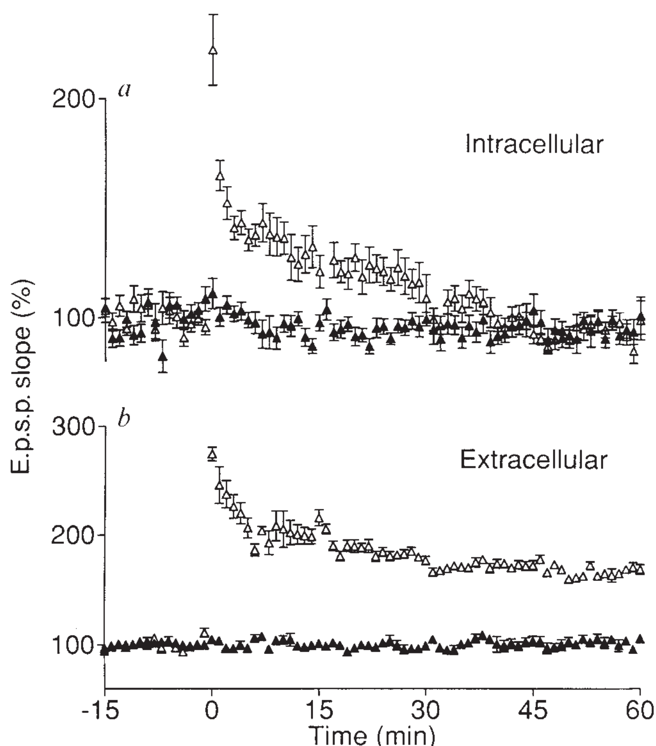

FIG. 3 CBP blocks LTP but does not affect normal synaptic transmission. Two independent pathways S1 (△) and S2 (▲) synapsing on the same cell (*a*) or population of cells (*b*) were alternatively stimulated at 0.1 Hz. Standard LTP-inducing tetani were applied to the S1 pathway while stimulation of the S2 pathway was halted. Graphs are a summary of 4 experiments. No difference was observed when cells were recorded with microelectrodes containing 1.9 mM CBP ($n=2$) or 190 μM CBP ($n=2$).

found in isolated postsynaptic densities[13,14] and should have ready access to the rise in Ca^{2+} and subsequent activation of CaM due to NMDA receptor activation. It is also in an ideal position to modify postsynaptic glutamate receptors. Biochemical studies have demonstrated that following CaM-dependent autophosphorylation, CaM-KII no longer requires CaM to maintain its kinase activity[15,31]. It has recently been proposed that persistent kinase activity is required for the maintenance of LTP[21,23]. As has been predicted from modelling of the biochemical properties of CaM-KII, the initial activation of CaM by postsynaptic Ca^{2+} may permit CaM-KII to become constitutively active and serve as a mechanism for maintaining increased synaptic strength[15,16]. The simplest hypothesis to account for the increase in synaptic strength during LTP is that activation of CaM, kinase activity and perhaps as yet unknown biochemical processes, somehow modify the number and/or properties of the non-NMDA receptors which generate the e.p.s.p. at these synapses[20,32-34] although more complicated events cannot be ruled out[35-37]. □

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Thymic cortical epithelial cells lack full capacity for antigen presentation

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SEVERAL recent studies have suggested that interactions between thymocytes and thymic stromal cells are essential for the development and elimination of antigen-reactive T lymphocytes[1–4]. It is important, therefore, to characterize the stromal cells involved in presentation of antigen in the thymus. In a previous report, we demonstrated, using T-cell hybridomas, that three distinct types of antigen presenting cells in the thymus (cortical epithelial cells, macrophages, and dendritic cells) constitutively expressed self haemoglobin/Ia complexes[5]. Here we report that one of these cell types, the cortical epithelial cell, does not induce stimulation of T-lymphocyte clones even though the antigen/Ia complex required for antigen-specific recognition is present. This lack of response occurs with both T_{H1} and T_{H2} clones. Responsiveness of the T_{H2} clone can be restored by adding the murine lymphokine interleukin-1β to the culture system.

T-cell hybridomas are known to be stimulated by the engagement of their T-cell receptor (TCR) with antigen/Ia complexes. By contrast, freshly isolated T-cell clones require in addition the presence of one or more secondary molecules, such as adhesion or co-stimulatory proteins, for T-cell stimulation to occur. To fully define the antigen presenting capabilities of thymic stromal cells, it is important to discover whether they express these additional molecules. For this investigation, two haemoglobin (Hb)-specific, $CD4^+$, $I\text{-}E^k$-restricted T-cell clones, PL17 and HS8 were used. On stimulation, these T-cell clones secreted interleukin-2 (IL-2) and γ-interferon but not IL-4, and were therefore classified as belonging to the T_{H1} subset of murine helper T-cell clones[6]. As observed with murine Hb-specific hybridomas, the Hb-specific T-cell clones responded to splenic antigen presenting cells (APCs) directly after removal of the spleens from the mouse, in the absence of any exogenous antigen (Fig. 1).

We have previously demonstrated that three types of APCs in the thymus, cortical epithelial cells, macrophages, and dendritic cells, can stimulate Hb-specific T-cell hybridomas[5]. The Hb-specific T-cell clones were tested, therefore, for reactivity towards these three types of APCs. The results (Fig. 2*a*) clearly show that thymic nurse cells (TNC), the *in vitro* correlate of cortical epithelial cells[7–9], did not stimulate the HS8 clone to proliferate, even though the TNCs had sufficient Hb/Ia complexes to stimulate the YO1.6 hybridoma (Fig. 2*a*, top). The other two populations of APCs, the Mϕ/DC and the T-ROS (a representative sample of Ia^- macrophages and Ia^+ dendritic cells, which when isolated have rosetted thymocytes surrounding them) were both fully capable of stimulating the HS8 clone (Fig. 2*a*, bottom). Identical results were seen using the PL17 clone (data not shown). As shown in Fig. 2*b*, equivalent results, with a limited-response to the thymic cortical epithelial cell population, were found when a hen egg-white lysozyme (HEL)-specific T_{H2} clone, CIB4 (ref. 10), was used as the responding cell. No

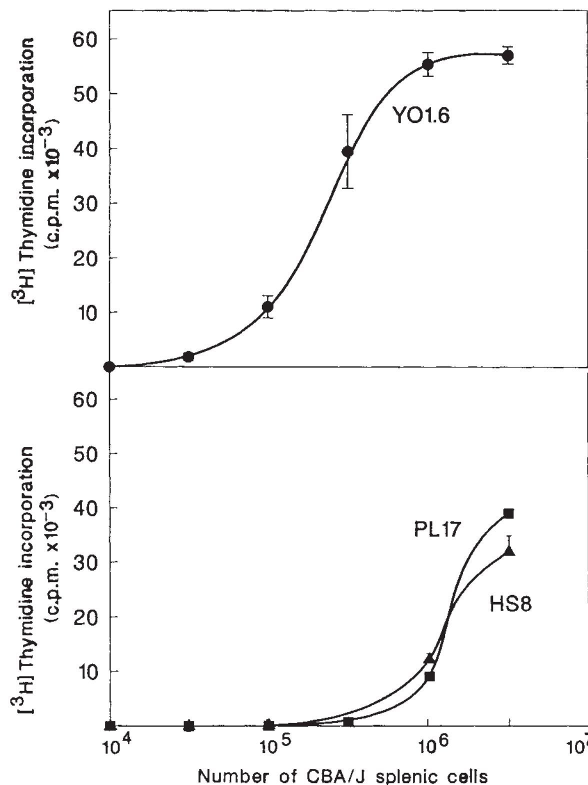

FIG. 1 Stimulation of the Hb-specific YO1.6 hybridoma (top) and the Hb-specific PL17/HS8 clones (bottom) by CBA/J splenic APCs. It should be noted that the PL17 and HS8 clones recognize the same peptide antigen $Hb^{\beta dminor}$(62–76) as the YO1.6 hybridoma, and are approximately tenfold less sensitive to the *in vitro* Hb/Ia complexes than the YO1.6 hybridoma. No stimulation of either the YO1.6 hybridoma or the PL17/HS8 clones was seen when splenic cells isolated from mice with the non-stimulatory Hb haplotype (Hbb^s, $H\text{-}2^k$) were used.

METHODS. The stimulation of the Hb-specific T-cell clones was quantiated by measuring the [^{3}H] thymidine incorporation of the proliferating T-cell clone (1×10^4 per well) in response to irradiated CBA/J splenic APCs (3,000 rads). [^{3}H] Thymidine was added at 72 h and the cells were collected 24 h later. The hybridoma stimulation assay was as described[16], with the exception that a single cell suspension of irradiated splenic cells was used as the APC population. No exogenous antigen was added to the culture system. The specificity of the YO1.6 hybridoma is as described[16]. The $I\text{-}E^k$-restricted Hb-specific T-cell clones were generated by immunizing CE/J mice (Hbb^s) with 20 μg hemolysate from CBA/J mice (Hbb^d) emulsified in complete Freund's adjuvant (Difco H37Ra). Seven days later the draining lymph nodes were removed, a single cell-suspension was prepared and incubated in upright T-25 flasks (Corning) at 1×10^7 cells ml^{-1} with 100 $\mu g\ ml^{-1}$ CBA/J hemolysate in 5 ml RPMI 1640 supplemented with 10% horse serum, 2 mM L-glutamine and 50 $\mu g\ ml^{-1}$ gentamicin. Four days later, the T-cell blasts were isolated on a Ficoll-Hypaque gradient (Pharmacia) and recultured at 1×10^5 cells ml^{-1} with irradiated (3,000 rads) CE/J spleen filler cells ($5 \times 10^6\ ml^{-1}$) in media containing 50 μM 2-mercaptoethanol and 10% FCS instead of horse serum. Ten days later, the viable cells were recovered and recultured with $5 \times 10^6\ ml^{-1}$ irridiated CBA/J filler cells at 1×10^5 cells ml^{-1}. This bulk line was passed every 14 days on fresh CBA/J filter cells. At the end of the second biweekly cycle, the bulk line was subcloned in microtitre wells containing 20% rat con-canavalin A (con A) supernatant and 1×10^6 CBA/J filler cells per well. Positive wells were expanded on fresh filler cells and weaned off rat con A supernatant. These T-cell clones were maintained on a cycle of 4 day stimulation on CBA/J (Hbb^d) filler cells and 10 day rest on B10.BR/SgSnJ (Hbb^s) filler cells and 0.5% rat con A supernatant.

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