ing activity. This would be analogous to the extracellular functions of ATP or cyclic adenosine monophosphate (*28–30*), which are very much different from their intracellular roles. Further experimentation will be required to clarify these various possibilities and to fully elucidate the contribution of this lymphocyte-associated ADP–ribosyl cyclase activity in the immune system.

REFERENCES AND NOTES

1. E. L. Reinherz, P. C. Kung, G. Goldstein, R. H. Levey, S. F. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1588 (1980).
2. F. Malavasi *et al.*, *Int. J. Clin. Lab. Res.* **22**, 73 (1992).
3. L. Santos-Argumedo, C. Teixeira, G. Preace, P. A. Kirkham, R. M. E. Parkhouse, *J. Immunol.* **151**, 3119 (1993).
4. N. Harada *et al.*, *ibid.*, p. 3111.
5. D. G. Jackson and J. I. Bell, *ibid.* **144**, 2811 (1990).
6. M. R. Hellmich and F. Strumwasser, *Cell Regul.* **2**, 193 (1991).
7. H. C. Lee and R. Aarhus, *ibid.*, p. 203.
8. D. L. Glick *et al.*, *ibid.*, p. 212.
9. D. J. States, T. F. Walseth, H. C. Lee, *Trends Biochem. Sci.* **17**, 495 (1992).
10. M. Howard *et al.*, data not shown.
11. H. C. Lee, T. F. Walseth, G. T. Bratt, R. N. Hayes, D. L. Clapper, *J. Biol. Chem.* **264**, 1608 (1989).
12. A. Galione, H. C. Lee, W. B. Busa, *Science* **253**, 1143 (1991).
13. S. Takasawa, K. Nata, H. Yonekura, H. Okamoto, *ibid.* **259**, 370 (1993).
14. L. G. Mészáros, J. Bak, A. Chu, *Nature* **364**, 76 (1993).
15. H. C. Lee, *J. Biol. Chem.* **268**, 293 (1993).
16. P. J. Dargie, M. C. Agre, H. C. Lee, *Cell Regul.* **1**, 279 (1990).
17. H. Koshiyama, H. C. Lee, A. H. Tashijian Jr., *J. Biol. Chem.* **266**, 16985 (1991).
18. K. Currie, K. Swann, A. Galione, R. H. Scott, *Mol. Biol. Cell* **3**, 1415 (1992).
19. M. A. White, S. P. Watson, A. Galione, *FEBS Lett.* **318**, 259 (1993).
20. Soluble CD38-FLAG was constructed with the polymerase chain reaction (PCR) to amplify the murine CD38 extracellular domain commencing with amino acid residue 46. The accuracy of the PCR-derived recombinant CD38 construct was confirmed by sequencing. The CD38 PCR fragment was ligated into a pMeV18S derivative containing a CD8 leader sequence adjacent to an eight–amino acid FLAG sequence [T. P. Hopp *et al.*, *BioTechnology* **6**, 1204 (1988)]. Expression of this construct in mammalian cells yielded a polypeptide that contained the entire extracellular domain of murine CD38, together with the FLAG octapeptide (NH_2-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH) attached at the NH_2-terminus. The expressed material was detected by enzyme-linked immunosorbent assay with plates (96 well) coated with mouse monoclonal antibody to FLAG (International Biotechnologies, New Haven, CT). Bound CD38 was detected with anti-CD38.
21. Supernatants were concentrated and affinity-purified with a FLAG antibody column (International Biotechnologies, New Haven, CT). The protein was eluted with 0.1 M glycine-HCl (pH 3.0), then further purified on an HPLC Mono-Q column (Pharmacia) with a salt gradient of 10 mM tris-HCl (pH 7.5) and 2 M NaCl in 10 mM tris-HCl (pH 7.5). The resultant material was composed of a single protein species of approximately 40 kD after SDS–polyacrylamide gel electrophoresis.
22. H. C. Lee, R. Aarhus, T. F. Walseth, *Science* **261**, 352 (1993).
23. H. C. Lee and R. Aarhus, *Biochim. Biophys. Acta* **1164**, 68 (1993).
24. I. M. Serventi, J. Moss, M. Vauhan, *Curr. Top. Microbiol. Immunol.* **175**, 43 (1992).
25. L. Stryer, *Biochemistry* (Freeman, New York, ed. 3, 1988), p. 191.
26. J. C. Cambier and J. T. Ransom, *Annu. Rev. Immunol.* **5**, 175 (1987).
27. H. Yamada *et al.*, *J. Exp. Med.* **177**, 1614 (1993).
28. C. D. Benham, *Nature* **359**, 103 (1992).
29. F. A. Edwards, A. J. Gibb, D. Colquhoun, *ibid.*, p. 144.
30. R. L. Johnson *et al.*, *J. Biol. Chem.* **267**, 4600 (1992).

31. We thank D. M. Finn for manuscript preparation. DNAX Research Institute is fully supported by Schering Plough Corporation. This study was partially supported by NIH grants HD17484 (H.C.L.) and DA05695 (T.F.W.).

4 May 1993; accepted 14 September 1993

Immune Responses in Interleukin-2–Deficient Mice

Thomas M. Kündig,* Hubert Schorle, Martin F. Bachmann, Hans Hengartner, Rolf M. Zinkernagel, Ivan Horak

The role of costimulatory signals in T cell induction was evaluated in mice lacking the interleukin-2 (IL-2) gene. In vitro secondary antiviral T cell responses were absent unless IL-2 was added, confirming the crucial role of IL-2 in vitro. In vivo, primary and secondary cytotoxic T cell responses against vaccinia and lymphocytic choriomeningitis virus were within normal ranges. B cell reactivity to vesicular stomatitis virus was not impaired. T helper cell responses were delayed but biologically functional. Natural killer cell activity was markedly reduced but inducible. These normal in vivo immune responses in IL-2–deficient mice question the importance of IL-2 as defined by in vitro studies.

Stimulation of the T cell receptor (TCR) alone is insufficient for T cell induction; a second, costimulatory signal must be provided (*1*). The interaction between B7 and BB1 (*2*) on the antigen-presenting cell with CD28 (*3*) and CTLA-4 (*4*) on the T cell can provide this second signal. This costimulation drastically increases the production of IL-2 (*5*), which is crucial for in vitro proliferation of T cells (6) and is assumed to be the central growth hormone of the immune system. IL-2 is produced mainly by activated T helper (T_H) cells (*7*) and stimulates their proliferation in an autocrine fashion. IL-2 is also required for in vitro growth of cytotoxic T cell (CTL) lines and has been shown to enhance natural killer (NK) cell (8) and B cell (9) responses. In a study to assess the importance of IL-2 in immune responsiveness in vivo, mice made deficient for the IL-2 gene by homologous recombination (*10*) were evaluated for immune responses against three different viruses. CTL function was studied with the use of vaccinia virus and lymphocytic choriomeningitis virus (LCMV), since cytotoxic responses are crucial in resolving these two viral infections (*11*). Humoral immune responsiveness was assessed with vesicular stomatitis virus (VSV) as a model antigen where B cells and T_H cells play the major role in the immune response (*12*).

T. M. Kündig, M. F. Bachmann, H. Hengartner, R. M. Zinkernagel, Institute of Experimental Immunology, Schmelzbergstr. 12, University of Zürich, Zürich, Switzerland.

H. Schorle and I. Horak, Institute for Virology and Immunobiology, Versbacherstr. 7, University of Würzburg, Würzburg, Germany.

*To whom correspondence should be addressed.

It is not clear whether CTLs can be generated in the absence of IL-2. In many model systems, the generation of CTL responses depends on the presence of T_H cells (*13*), which are the major source of IL-2 (*7*), although certain viruses [for example, ectromelia (*14*), LCMV (*15*), and vaccinia virus (*16*)] can induce an efficient CTL response in the absence of T_H cells. In these latter cases, IL-2 from $CD8^+$ T cells rather than T_H cells has been assumed to be involved (*17*).

Vaccinia-specific CTL responses were measured 6 days after intravenous infection with vaccinia virus in three independent experiments. IL-$2^{-/-}$ mice and IL-$2^{+/-}$ littermates or C57BL/6 mice responded equally well (Fig. 1C).

LCMV-specific cytotoxicity was assessed 9 days after infection by primary ^{51}Cr-release assays. The frequency of LCMV-specific CTLs generated by IL-$2^{-/-}$ mice was reduced about threefold as compared with that generated by heterozygous littermates in both independent experiments (Fig. 1, A and B). This decrease in specific CTL frequency in the latter assay was of no biological importance, since the CTL responses in IL-$2^{-/-}$ mice mediated a normal early immunopathological swelling-reaction of the footpads starting 7 to 8 days after local infection with LCMV (Fig. 1, D and E). The early phase of swelling is dependent exclusively on $CD8^+$ T cells (*15*, *18*). The primary CTL response eliminated LCMV below detection levels from spleens and livers by day 10 in both IL-$2^{-/-}$ and control mice (*19*). IL-$2^{-/-}$ mice that had been primed intravenously with LCMV were protected against lethal choriomeningitis

after intracerebral infection with LCMV, whereas unprimed control mice died (*19*). Protection against lethal choriomeningitis is mediated exclusively by $CD8^+$ memory CTLs (*20*). Thus, primary and secondary CTL responses can be said to have remained within normal ranges, despite the lack of IL-2.

The data shown are representative for specific CTL responses that could be induced in eight out of eight healthy IL-$2^{-/-}$ mice aged 4 to 6 weeks. Four additional IL-$2^{-/-}$ mice, older than 8 weeks, developed complex disease symptoms resulting in death (*21*); these mice were cachectic, had either tiny or huge spleens, and failed to generate measurable CTL responses after infection.

In contrast to the functional secondary CTL responses in vivo, in vitro stimulation of primed vaccinia-specific memory CTLs from IL-$2^{-/-}$ mice did not generate cytotoxicity (Fig. 2B). CTL activity could be restored by addition of either concanavalin A (Con A) supernatant (containing IL-2) (Fig. 2C) or recombinant IL-2 (Fig. 2D), but not by addition of recombinant IL-1, IL-4, or IL-6, alone or in combination, tested at 2.5 or 25 U/ml (*19*). Similar results were obtained for LCMV-specific secondary responses in vitro (*19*).

Previous studies have demonstrated that IL-4 (*22*), and possibly other interleukins (*23*), can substitute for some of the IL-2 activities in vitro. However, in our in vitro system the lymphokines tested above could not replace IL-2. This suggests that, in vivo, either a complex network of cytokines, which apparently may not be stimulated readily in vitro, or cytokines other than those tested replace IL-2 in IL-$2^{-/-}$ mice.

The ability of IL-$2^{-/-}$ mice to mount a neutralizing immunoglobulin (Ig) response was assessed after intravenous infection with VSV (Fig. 3). The IgM response to VSV, which is independent of T_H cells (*15, 24*), was comparable in IL-$2^{-/-}$ and IL-$2^{+/-}$ mice, suggesting a normal function and induction of B cells. The Ig class switch from IgM to IgG is strictly T_H cell–dependent (*15, 24*). The delayed IgG response in the IL-$2^{-/-}$ mice therefore demonstrated an impaired but nevertheless efficient function of $CD4^+$ T_H cells. IgG subclass distribution was within normal ranges (*19*).

NK activity was measured on day 3 after high-dose intravenous infection with

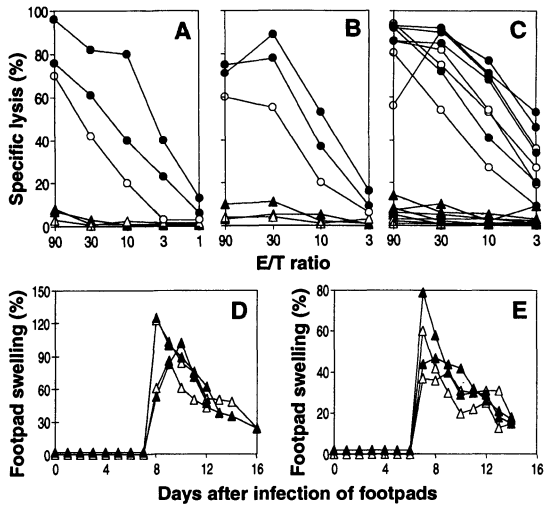

Fig. 1. LCMV- and vaccinia virus–specific cytotoxicity and LCMV-induced footpad-swelling reactions. IL-$2^{-/-}$ (open symbols) and IL-$2^{+/-}$ or C57BL/6 (closed symbols) mice were infected with (**A**) LCMV strain WE [200 plaque-forming units (PFU) given intravenously, day 9] (**B**), LCMV strain Armstrong (300 PFU, day 9), or (**C**) vaccinia WR (2×10^6 PFU, day 6) . The mice were tested for cytotoxicity on MC57 target cells infected with the respective virus (circles) or left uninfected (triangles). In (C), results from three independent experiments are shown. Each curve represents values taken from one mouse. ^{51}Cr-release was measured in duplicate, and mean values are shown (SD < 12% for all values). Spontaneous ^{51}Cr-release was less than 20% for all targets. (**D** and **E**) LCMV-induced footpad-swelling reaction. IL-$2^{-/-}$ (open triangles) and IL-$2^{+/-}$ littermates or C57BL/6 mice (closed triangles) were injected into both hind footpads on day 0 with 500 PFU of LCMV strain WE (D) or 1000 PFU of LCMV strain Armstrong (E), in a volume of 30 μl. Subsequent footpad swelling was measured with a spring-loaded caliper and is given as the percentage of footpad thickness before infection. Each curve shows the average swelling of both hind footpads of one mouse (SD < 12%).

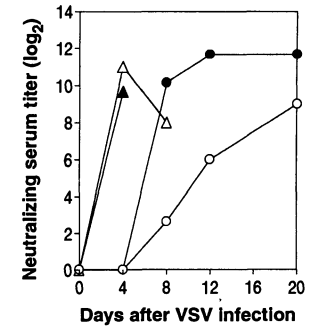

Fig. 3. Neutralizing antibody response to VSV. IL-$2^{-/-}$ (open symbols) and IL-$2^{+/-}$ (closed symbols) mice were infected intravenously with VSV serotype New Jersey on day 0 (2×10^6 PFU). Titers of neutralizing IgM (triangles) and of neutralizing IgG (circles) were determined in sera in groups of three mice on days 4, 8, 12, and 20 as described (*15*). Mean titers are shown as log_2 of 40-fold prediluted sera.

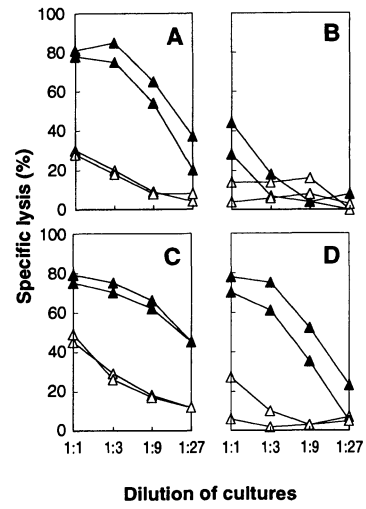

Fig. 2. Generation of secondary antiviral CTLs in vitro from virus-primed spleen cells of IL-$2^{-/-}$ mice. (**A**) C57BL/6 mice or (**B** to **D**) IL-$2^{-/-}$ mice were infected intravenously with 2×10^6 PFU of vaccinia WR on day 6. Spleen cells were then restimulated by vaccinia WR–infected spleen cells. In (A) and (B) no cytokines were added to the culture medium, in (C) 5% Con A supernatant was added, and in (D) recombinant IL-2 (25 U/ml) was added. ^{51}Cr-release was done on MC57 cells infected with vaccinia virus (closed triangles) or left uninfected (open triangles). These results are also representative for a second independent experiment and a comparable experiment with LCMV-primed responder cells. Each curve represents values taken from one mouse. ^{51}Cr-release was measured in duplicate, and mean values are shown (SD < 13% for all values). Spontaneous ^{51}Cr-release was less than 20% for all targets.

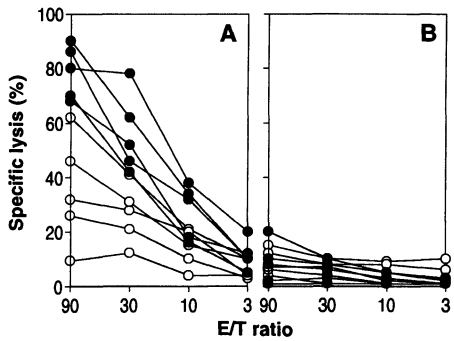

Fig. 4. NK activity in IL-$2^{-/-}$ (open circles) and C57BL/6 control (closed circles). Mice were infected intravenously with a high dose of LCMV strain WE (2×10^6 PFU) 3 days previously. Specific lysis was measured as ^{51}Cr-release on (**A**) NK-susceptible YAC and (**B**) NK-resistant P815 target cells. Pooled results from three independent experiments are shown. Each curve represents values taken from one mouse. ^{51}Cr-release was measured in duplicate, and mean values are shown (SD < 13% for all values). Spontaneous ^{51}Cr-release was less than 25% for all targets.

LCMV (Fig. 4) (25). Lysis of NK-sensitive yeast artificial chromosome (YAC) targets was reduced by a factor of 3 to 9 in IL-$2^{-/-}$ mice as compared with control mice.

The fact that NK cell responses were impaired but inducible in IL-2–deficient mice confirms the previous finding that IL-2 supports proliferation of NK cells (8) but that interferon-α (IFN-α) and IFN-β may be more important in the induction of the NK cell response (26).

Taken together, in vivo CTL responses against vaccinia virus and LCMV as well as B and T_H cell responses against VSV were normal or only slightly impaired. Not only were antigen-specific CTL and T_H cells readily induced, but in vivo proliferation occurred: In uninfected mice, the frequency of vaccinia- or LCMV-specific CTLs is less than 10^{-6} spleen cells. During infection, this frequency increases as a result of proliferation. For primary ex vivo cytotoxicity to be observed, the frequency of virus-specific CTLs must reach the range of 10^{-2} (20, 27). Thus, the fact that primary in vivo cytotoxicity was generated in IL-2–deficient mice suggests that proliferation on the order of 10,000 has occurred despite the absence of IL-2. The mildness of the effects on in vivo T and B cell responses against the viruses tested was unexpected. The notion that IL-2 plays a central and limiting role in immunoregulation is based mostly on studies in vitro and is confirmed here for secondary CTL responses in vitro. IL-2 receptor γ chain mutations have recently been shown to result in severe combined immunodeficiency in humans (28). In contrast to the IL-$2^{-/-}$ mice, T cells in these patients are diminished in numbers and are apparently unresponsive. These differences suggest either that IL-2 has a more important role in human T cell development and function or that the IL-2 receptor γ chain has additional functions, for example, as a component of other cytokine receptors.

In conclusion, in vivo immune responses in IL-2–deficient mice question the importance of IL-2 as defined by in vitro studies. It appears that, in vivo, other lymphokines may compensate for the IL-2 defect.

REFERENCES AND NOTES

1. R. H. Schwartz, *Science* **248**, 1349 (1990); *Cell* **71**, 1065 (1992); T. Taniguchi and Y. Minami, *ibid.* **73**, 5 (1993).
2. G. J. Freeman *et al.*, *J. Exp. Med.* **174**, 625 (1991); L. Chen, *et al.*, *Cell* **71**, 1093 (1992).
3. P. S. Linsley *et al.*, *J. Exp. Med.* **173**, 721 (1991).
4. J.-F. Brunet *et al.*, *Nature* **328**, 267 (1987).
5. T. Lindsten, C. H. June, J. A. Ledbetter, G. Stella, C. B. Thompson, *Science* **244**, 339 (1989); G. R. Crabtree, *ibid.* **243**, 355 (1989); J. D. Fraser, B. A. Irving, G. R. Crabtree, A. Weiss, *ibid.* **251**, 313 (1991).
6. D. A. Morgan, F. W. Ruscetti, R. C. Gallo, *ibid.* **193**, 1007 (1976); S. Gillis and K. A. Smith, *Nature* **268**, 154 (1977).
7. S. C. Meuer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1509 (1984); H. Wagner and M. Röllinghof, *J. Exp. Med.* **148**, 1523 (1978).
8. C. A. Biron, H. A. Young, M. T. Kasaian, *J. Exp. Med.* **171**, 173 (1990); Y. Ishida *et al.*, *ibid.* **170**, 1103 (1989).
9. P. Bretscher and M. Cohn, *Science* **169**, 1042 (1970); H. N. Claman, S. D. Miller, P. J. Conlon, J. W. Moorhead, *Adv. Immunol.* **30**, 121 (1980); G. J. W. Nossal, *Annu. Rev. Immunol.* **1**, 33 (1983).
10. H. Schorle, T. Holtschke, T. Hünig, A. Schimpl, I. Horak, *Nature* **352**, 621 (1991).
11. C. A. Mims, *The Pathogenesis of Infectious Disease* (Academic Press, London, 1987); R. V. Blanden, *Transplant. Rev.* **19**, 56 (1974).
12. R. R. Wagner, *The Rhabdoviruses* (Plenum, New York, 1987); L. Lefrancois, *J. Virol.* **51**, 208 (1984).
13. G. Gromo, R. L. Geller, L. Inverardi, F. H. Bach, *Nature* **327**, 424 (1987); J. R. Bennink and P. C. Doherty, *ibid.* **276**, 829 (1978); R. B. Ashman and A. Müllbacher, *J. Exp. Med.* **150**, 1277 (1979); R. M. Zinkernagel *et al.*, *ibid.* **147**, 897 (1978); W. M. Kast, A. M. Bronkhorst, L. P. DeWaal, C. J. M. Melief, *ibid.* **164**, 723 (1986).
14. R. M. L. Buller, K. L. Holmes, A. Hügin, T. N. Frederickson, H. C. Morse III, *Nature* **328**, 77 (1987).
15. T. P. Leist, S. P. Cobbold, H. Waldmann, M. Aguet, R. M. Zinkernagel, *J. Immunol.* **138**, 2278 (1987).
16. T. P. Leist, M. Kohler, R. M. Zinkernagel, *Scand. J. Immunol.* **30**, 679 (1989).
17. M. T. Kasaian and C. A. Biron, *J. Immunol.* **142**, 1287 (1989); T. Mizouchi, A. W. Hügin, H. C. Morse III, A. Singer, M. L. Buller, *ibid.*, p. 270.
18. D. Moskophidis and F. Lehmann-Grube, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3291 (1989).
19. T. M. Kündig *et al.*, unpublished data.
20. S. Oehen, H. P. Waldner, T. M. Kündig, H. Hengartner, R. M. Zinkernagel, *J. Exp. Med.* **176**, 1273 (1992).
21. B. Sadlack *et al.*, *Cell*, in press.
22. M. B. Widmer, R. B. Acres, H. M. Sassenfeld, K. H. Grabstein, *J. Exp. Med.* **166**, 1447 (1987); A. O'Garra *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5228 (1986); R. Fernandez-Botran *et al.*, *ibid.*, p. 9689; E. A. Kurt-Jones, S. Hamberg, J. Ohara, W. E. Paul, A. K. Abba, *J. Exp. Med.* **166**, 1774 (1987).
23. K. Takatsu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4234 (1987).
24. S. Charan and R. M. Zinkernagel, *J. Immunol.* **136**, 3057 (1986); S. Charan, A. W. Hügin, A. Cerny, H. Hengartner, R. M. Zinkernagel, *J. Virol.* **57**, 1139 (1986); R. M. Zinkernagel, S. Cooper, J. Chambers, R. A. Lazzarini, H. Hengartner, *Nature* **344**, 68 (1990).
25. M. J. Buchmeier, R. M. Welsh, F. J. Dutko, M. B. A. Oldstone, *Adv. Immunol.* **30**, 275 (1980).
26. M. T. Kasaian and C. A. Biron, *J. Exp. Med.* **171**, 745 (1990).
27. D. Moskophidis, U. Assmann Wischer, M. M. Simon, F. Lehmann Grube, *Eur. J. Immunol.* **17**, 937 (1987).
28. M. Noguchi *et al.*, *Cell* **73**, 247 (1993).

29. We thank H. Haber and A. Peters for assistance. Supported by the Swiss National Science Foundation, the Deutsche Forschungsgemeinschaft (SFB165), and the Fonds der Chemischen Industrie.

17 May 1993; accepted 23 August 1993

A Ca-Dependent Early Step in the Release of Catecholamines from Adrenal Chromaffin Cells

Ludolf von Rüden* and Erwin Neher†

Intense stimuli, such as trains of depolarizing pulses or the caffeine-induced release of calcium from intracellular stores, readily depress the secretory response in neuroendocrine cells. Secretory responses are restored by rest periods of minutes in duration. This recovery was accelerated when the concentration of cytosolic calcium was moderately increased and probably resulted from calcium-dependent replenishment of a pool of release-ready granules. Continuously increased concentrations of calcium led to the overfilling of such a pool. Subsequently, secretory responses to stronger calcium stimuli were augmented. Hormone-induced calcium transients with a plateau phase of increased concentration of calcium may enhance the secretory response in this way.

The docking of secretory vesicles at active zones is common to most neuronal synapses (1). It allows for rapid secretion during synaptic transmission. Recent evidence suggests that neuroendocrine cells also have a small pool of granules that can be released more quickly than the rest (2–6). When the intracellular concentration of Ca^{2+} $[Ca^{2+}]_i$ rises, secretion occurs with several distinct kinetic phases (5, 6). We described a model (7) that can simulate different types of secretory experiments under the simple assumption of a two-step process: Granules migrate during maturation from a reserve pool A to a release-ready pool B, the latter being the source of secreted vesicles. To accommodate all the available data, we had to assume that both steps in this scheme are influenced by $[Ca^{2+}]_i$. The model predicted that the size of the release-ready pool should increase with increasing $[Ca^{2+}]_i$ up to about 0.5 μM, resulting in the augmented secretion to subsequent stimuli. Such an augmentation has also been inferred from biochemical measurements (4) and from the amplitudes of kinetic components in experiments with caged calcium (6). Here we investigated the effects of $[Ca^{2+}]_i$ on the release-ready pool of vesicles

Department of Membrane Biophysics, Max-Planck-Institut für biophysikalische Chemie, Am Fassberg, 37077 Göttingen, Germany.

*Present address: Departmento de Farmacologia y Terapéutica, Faculdad de Medicina, Universidad Autonoma de Madrid, 28029 Madrid, Spain.

†To whom correspondence should be addressed.