of obesity when caused by an insufficiency or deficiency of the adipose hormone. Finally, during the revision process, three reports were published[9-11], also demonstrating the antiobesity effects of OB in *ob ob* mice. □

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# Isolation and structure of the endogenous agonist of opioid receptor-like $ORL_1$ receptor

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**THE $ORL_1$ receptor, an orphan receptor whose human[1] and murine[2-8] complementary DNAs have recently been characterized, structurally resembles opioid receptors and is negatively coupled with adenylate cyclase[1]. $ORL_1$ transcripts are particularly abundant in the central nervous system. Here we report the isolation, on the basis of its ability to inhibit the cyclase in a stable recombinant CHO($ORL_1^+$) cell line, of a neuropeptide that resembles dynorphin A[9] and whose amino acid sequence is Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln. A rat-brain cDNA encodes the peptide flanked by Lys-Arg proteolytic cleavage motifs. The synthetic heptadecapeptide potently inhibits adenylate cyclase in CHO($ORL_1^+$) cells in culture and induces hyperalgesia when administered intracerebroventricularly to mice. Taken together, these data indicate that the newly discovered heptadecapeptide is an endogenous agonist of the $ORL_1$ receptor and that it may be endowed with pro-nociceptive properties.**

Human[1] and murine[2-8] cDNAs have been identified that encode $ORL_1$ (Opioid Receptor-Like 1), a new G-protein-coupled receptor whose amino acid sequence is most closely related to those of opioid receptors. The orphan receptor mediates inhibition of forskolin-induced accumulation of cAMP by the opiate etorphine in a stable recombinant CHO($ORL_1^+$) cell line[1]. This effect is exerted neither by the endogenous opioid peptides β-endorphin, enkephalins and dynorphins, nor by selective μ-, δ- and κ-opioid agonists[1,5,6]. Furthermore, etorphine is about three orders of magnitude less potent in inhibiting the cyclase by means of the $ORL_1$ than by an opioid receptor.

$ORL_1$ transcripts are present in the mouse and rat central nervous system (CNS)[1-6,8], as well as in a few peripheral organs such as intestine, vas deferens and spleen[5]. In the CNS these transcripts are most abundant in limbic areas, hypothalamus, pons and spinal cord, suggesting that $ORL_1$ plays a role in many central processes, including learning and memory, attention and emotions, homeostatis and sensory perception. The potential widespread importance of $ORL_1$ in neurophysiology and, possibly, neurophysiopathology, led us to identify an endogenous ligand of this receptor.

The structural homology of $ORL_1$ with opioid receptors, in particular the acidic extracellular loop 2 which, in the case of the κ-opioid receptor, is required for high-affinity binding of dynorphins[10,11], suggested that the ligand might be a peptide that resembled a dynorphin. Therefore, we used an extraction procedure previously used[12] to isolate a pituitary peptide that was later identified as dynorphin A (ref. 9). Because the orphan $ORL_1$ receptor is negatively coupled with adenylate cyclase, the desired compound was expected to inhibit forskolin-induced accumulation of cAMP in the recombinant CHO($ORL_1^+$) but not in the non-recombinant CHO($ORL_1^-$) cell line, as previously shown for etorphine[1].

Size-exclusion chromatography of the crude peptide extract on Bio-Gel P-2 proved efficient in revealing the desired activity (Fig. 1*a*). The active fractions were recovered in the void volume (pool F1) and, to some extent, in pool F2 (not shown), indicating that the biologically active substance had a relative molecular mass ($M_r$) of around 1,800, which is the nominal exclusion limit of Bio-Gel P-2. The other pools were either inactive or equally effective in inhibiting (or stimulating in the case of F7) adenylate cyclase in the two CHO cell lines (data not shown). Pool F1 was further purified by cation-exchange fast protein liquid chromatography (FPLC) (Fig. 1*b*). Activity was recorded in consecutive 1-ml fractions eluted at about 0.4 M NaCl, indicating the strongly basic nature of the active compound(s). These two fractions were then applied directly onto a reversed-phase high-performance liquid chromatography (HPLC) column and gradient-eluted with acetonitrile (Fig. 1*c*) to yield enough material of sufficient purity for protein sequencing. This material was found to be a heptadecapeptide of average $M_r$ 1,810 whose sequence was determined to be Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln. This most closely resembles dynorphin A (Tyr-*Gly-Gly-Phe*-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-*Lys*-Trp-Asp-*Asn-Gln*)[9], and may therefore be anticipated to interact with the $ORL_1$ receptor as the latter does with the κ-opioid receptor[10,11,13]. In particular, it can be viewed as being made up of an amino-terminal Phe-Gly-Gly-Phe 'message' for biological activity, followed by a Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys 'address' for enhanced potency[13]. The 'address' contains all the basic amino acid residues that are anticipated to bind the acidic second exofacial loop of the $ORL_1$ receptor.

To ensure that the sequence in question was not that of a major contaminant, the heptadecapeptide was synthesized and the synthetic heptadecapeptide was found to be a potent inhibitor of forskolin-induced accumulation of cAMP in the recombinant CHO($ORL_1^+$) cell line (Fig. 2). Its 50% inhibitory concentration ($IC_{50}$) was $0.9 \times 10^{-9}$ mol $l^{-1}$ and maximal inhibition was 90%. The synthetic peptide had no action on cyclase in the non-recombinant CHO($ORL_1^-$) cell line and displayed only micromolar or less potency in opioid receptor-mediated cyclase assays (data not shown).

The synthetic heptadecapeptide was active *in vivo* and affected reactivity to pain in the opposite manner to inhibition of $ORL_1$

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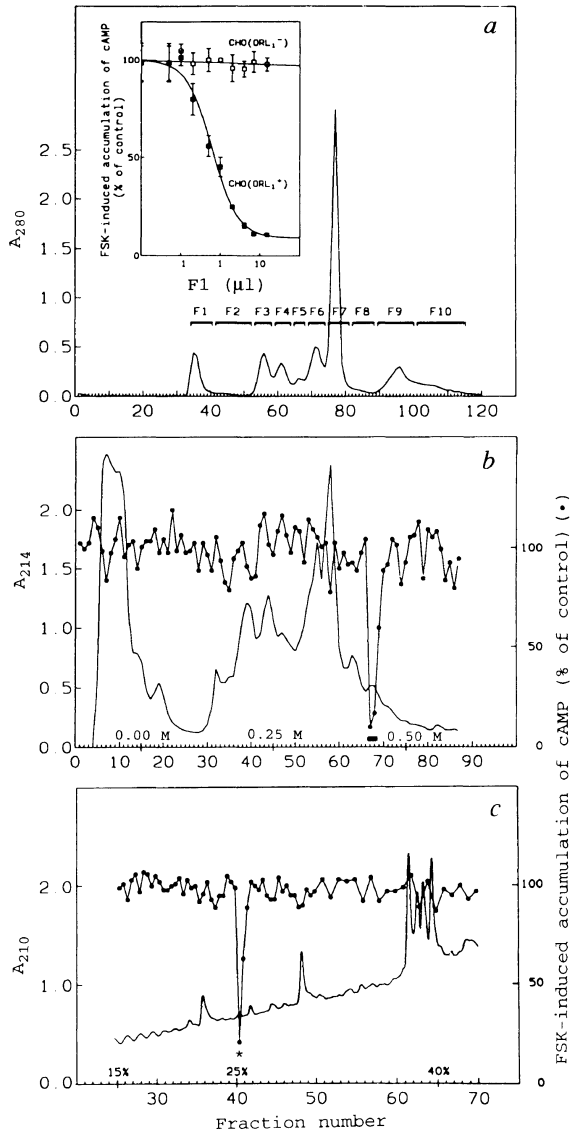


FIG. 1 Purification of the endogenous ligand of orphan receptor $ORL_1$. *a*, Size-exclusion chromatography of the crude peptide extract from rat brain. The insert shows that the sought-after activity, inhibition of FSK-induced synthesis of cAMP in CHO($ORL_1^+$) but not in CHO($ORL_1$) cells, is present in pool F1. *b*, Cation exchange FPLC of pool F1. The molarities above the *x*-axis are those of NaCl in the elution gradient. A major peak of activity was eluted at about 0.4 M NaCl (fractions 67 and 68, filled rectangles). *c*, Reversed-phase HPLC. The percentages above the *x*-axis are those of acetonitrile in the elution gradient. The desired biological activity, marked by an asterisk, was recovered as a single fraction which was eluted at 26% $CH_3CN$. FSK-induced accumulation of cAMP is shown (*b* and *c*) using filled circles.

METHODS. *a*, A crude extract[12] from freshly dissected and rapidly frozen rat brains (35 g) was lyophilized, dissolved in 10 ml 0.1 M AcOH and loaded onto pre-equilibrated Bio-Gel P-2 (2.3 × 90 cm column) at room temperature. Elution was at 0.6 ml $min^{-1}$. Fractions of 4 ml were collected, assayed for absorption at 280 nm and combined into pools F1–F10. Individual pools were lyophilized, dissolved in 2 ml phosphate buffer (20 mM $NaPO_4$, pH 6.4) and assayed for inhibition of forskolin-induced intracellular accumulation of cAMP in the CHO($ORL_1^+$) and CHO($ORL_1^-$) cell lines[1,17]. The CHO($ORL_1^+$) line used here is a more responsive subclone of the original recombinant clone[1]. Each data point is the mean±s.e.m. of triplicate determinations. *b*, Pool F1 (1.8 ml) was applied to a SP/8HR cation exchange column (Waters) equilibrated with 20 mM $NaPO_4$ buffer, pH 6.4 (buffer A). Elution was at 1 ml $min^{-1}$ with 100% buffer A for 15 min followed by a linear gradient from 100% buffer A to 50% buffer B (20 mM $NaPO_4$ + 1.0 M NaCl, pH 6.4) in 60 min. Each fraction (10 μl) was assayed for cyclase inhibitory activity. *c*, The active fractions (67 and 68) were pooled and loaded directly onto a reversed-phase HPLC column (μ Bondapak $C_{18}$, 3.9 × 300 mm; Waters). The eluents were 0.1% TFA in $H_2O$ (eluent A) and 0.1% TFA in acetonitrile (eluent B). Elution was at a flow rate of 1 ml $min^{-1}$ for 10 min with 100% A followed by a gradient to 10% B in 5 min and a gradient between 10% and 40% B in 50 min. Fractions of 0.5 ml were collected. Each fraction (10 μl) was dried (Speed Vac), redissolved in phosphate buffer and assayed for inhibition of cyclase in CHO($ORL_1^+$) cells. The molecular mass and sequence of the active compound in fraction 40.5 were determined with a Lasermat mass spectrometer (Finnigan MAT) and a model 476A protein sequencer (Applied Biosystems), respectively.

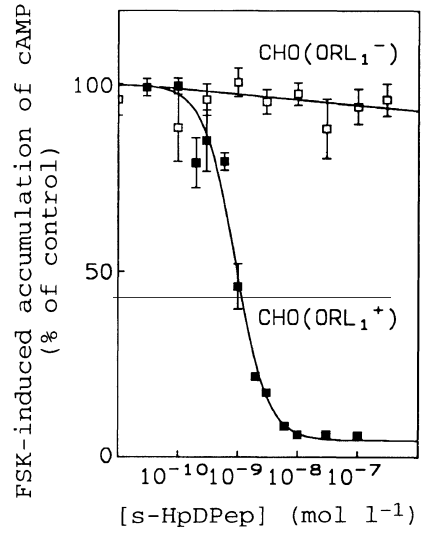


FIG. 2 Inhibition by the synthetic heptadecapeptide (s-HpDPep) of forskolin-induced accumulation of cAMP in recombinant CHO($ORL_1^+$) and non-recombinant CHO($ORL_1^-$) cells. Assay for cAMP in intact cells was as in the legend to Fig. 1. Each data point is the mean±s.e.m. of triplicate determinations.

receptor expression (Fig. 3). In a hot-plate test[14], mice that had been repeatedly treated with mAS[25,9], an antisense oligonucleotide[15] to $ORL_1$ mRNA, displayed substantially increased latencies to rearing and escape jumping in comparison with saline-treated animals: 38±2 s ($P<0.001$) versus 20±2 s, and 108±6 ($P<0.001$) versus 71±6 s, respectively (Fig. 3*a*). The 'missense' oligonucleotide hAS[25,9], the human counterpart of mAS[25,9], was totally ineffective in this respect. In another set of experiments, mice were intracerebroventricularly injected with 10 ng (5.5 pmol) or 100 ng (55 pmol) of the new peptide (Fig. 3*b*). At the larger dose of peptide, the animals showed significantly decreased latencies to rearing and escape jumping: 14±2 s (−36%, $P<0.01$) versus 22±2 s, and 48±2 (−26%, $P<0.001$) versus 65±3 s, respectively. In other words, the animals had become hyperreactive to nociceptive stimulation.

Final evidence that the new peptide must be physiologically significant and synthesized locally was provided by the isolation, from a rat-brain library, of a cDNA of 0.925 kb whose deduced amino acid sequence contains that of the heptadecapeptide (Fig. 4). The complete messenger RNA, estimated by northern blot analysis to be in the range 1.2–1.3 kb, is only slightly longer than the cDNA. Within the precursor, the heptadecapeptide sequence is flanked by Lys-Arg proteolytic excision motifs[16]. The precursor polypeptide contains additional pairs of basic amino acid residues that delineate other putative biologically active peptides, one of 35 amino acids immediately upstream and another of 17 amino acids immediately downstream of the heptadecapeptide. In this respect, the peptide's precursor would be more similar to pro-opiomelanocortin which is the precursor of several unrelated bioactive peptides than pro-enkephalin and pro-dynorphin, which each contain several copies of closely related (opioid) peptides[16].

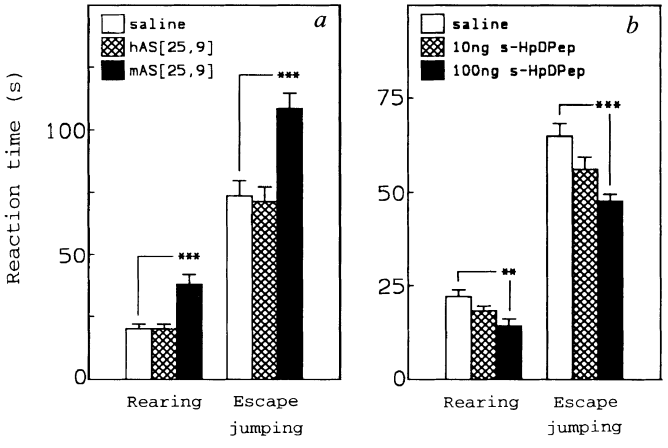


FIG. 3 Effects of repeated intracerebroventricular injection of antisense oligodeoxynucleotide (*a*) or a single injection of synthetic heptadecapeptide (*b*) on the latencies to rearing and escape jumping of mice in the hot-plate test[14]. Each bar represents the average reaction time ± s.e.m. from 20 animals. Statistical comparisons of oligo- or peptide- and saline (control)-treated groups were performed using the Student's *t* test. $^{**}P<0.01$, $^{***}P<0.001$.

METHODS. Male Swiss CD1 mice (20–25 g; Charles River) were used. *a*, Antisense oligonucleotide mAS[25,9] was the 17-mer 3′-GGAG-AAAGGACGGGGTA-5′ complementary to bases 9–25 of the translated region of the mouse $ORL_1$ mRNA. Control missense oligonucleotide hAS[25,9], was the 17-mer 3′-GGAGAAGGGGCGCGGCA-5′ complementary to bases 9–25 of the translated region of the human $ORL_1$ mRNA. The oligonucleotides were dissolved in sterile physiological saline (0.9 g $l^{-1}$ NaCl) at the final concentration of 2 mg $ml^{-1}$. The animals were manually injected with 10 µl of solution directly into the lateral brain ventricle every day for 4 consecutive days, and tested individually 96 h after the last injection. *b*, The animals were manually injected with 0 (control), 10 or 100 ng s-HpDPep in 10 µl of saline solution (9 g $l^{-1}$ NaCl) and tested 20 min later. The experiment was repeated twice with essentially the same results.

In summary, three important observations lend support to the claim that we have identified the naturally occurring agonist of receptor $ORL_1$: (1) a synthetic peptide of identical structure to the natural peptide exhibits nanomolar potency in the $ORL_1$-mediated cyclase inhibition assay; (2) the natural peptide shares structural similarity with other known peptides, in particular dynorphin A; and (3) the sequence of the natural peptide is present, framed by Lys-Arg proteolytic excision motifs, in a large

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001  CTCTCCAGCGTGTTCAGCAGCTGTCCCGAGGACTGCCTCACCTGCCAGGAGAGGCTCCAC 060
001   L  S  S  V  F  S  S  C  P  E  D  C  L  T  C  Q  E  R  L  H  020

061  CCGGCTCCGGGCAGCTTCAACCTGAAGCTGTGCATCCTCCAGTGTGAAGAGAAGGTCTTC 120
021   P  A  P  G  S  F  N  L  K  L  C  I  L  Q  C  E  E  K  V  F  040

121  CCCCGCCCTCTCTGGACTCTTTGCACCAAAGCCATGGCCAGTGACTCTGAGCAGCTCAGC 180
041   P  R  P  L  W  T  L  C  T  K  A  M  A  S  D  S  E  Q  L  S  060

181  CCTGCTGATCCAGAGCTCACGTCCGCTGCTCTTTACCAGTCGAAAGCCTCGGAGATGCAG 240
061   P  A  D  P  E  L  T  S  A  A  L  Y  Q  S  K  A  S  E  M  Q  080

241  CACCTGAAGAGAATGCCGCGTGTCAGGAGTGTGGTGCAAGCCCGAGACGCAGAGCCTGAG 300
081   H  L  [K  R] M  P  R  V  R  S  V  V  Q  A  R  D  A  E  P  E  100

301  GCAGATGCAGAGCCTGTCGCAGATGAGGCCGATGAGGTGGAGCAGAAGCAGCTGCAGAAA 360
101   A  D  A  E  P  V  A  D  E  A  D  E  V  E  Q  K  Q  L  Q  [K  120

361  AGGTTTGGGGGCTTCACTGGGGCCCGGAAGTCAGCCCGGAAGTTGGCCAACCAGAAGCGG 420
121   R] F  G  G  F  T  G  A  R  K  S  A  R  K  L  A  N  Q  [K  R] 140

421  TTCAGTGAGTTTATGAGGCAGTACCTGGTCCTGAGCATGCAGTCAAGCCAACGCCGGCGC 480
141   F  S  E  F  M  R  Q  Y  L  V  L  S  M  Q  S  S  Q  [R  R  R] 160

481  ACTCTGCACCAGAATGGTAATGTGTAGCCAGAAGGAGCCCCTCCCAGCTGCACCGGCCAC 540
161   T  L  H  Q  N  G  N  V  *                                   168

541  TGCAACCCATGAGCATCCAGGTGAGCCCCCGTACAGCATGTGTCCACACCAAGACCTGCA 600
601  GGCCGGGAGTCAGGATTCCTCCTTCCCTGAGGCACTGAACACCCGCGGCACCTCCCCACA 660
661  GCATGTCTCACCACAATCCTGTTGCTACATCAGAGTGTATTTTTGTAATTCCTCCAGCTA 720
721  ACATTTTAATGGCCCCATCTTCTTGCTCATCCTCTGCCCTCTCGTAGGGCCAGGTGAGAG 780
781  GAACATGAAATCAGACCTGGGGTTTTGCCTCACCACTGCCATAACTGGTTTGTAAAGGAG 840
841  CTGTTCTTTTTGACTGATTGTTTGAAACAACTTTCTCCATTAAACTTCTACTGAGCAAAA 900
901  TGGTTAATAAAAAAAAAAAAAAAA                                      925
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FIG. 4 Partial nucleotide and deduced amino acid sequences of the rat cDNA encoding the precursor of the novel brain peptide. The pairs of basic amino acid residues which are presumably implicated in enzymatic processing of the propeptide are boxed. The sequence of the new peptide is surrounded by two such motifs and underlined. The sequences of two other putative bioactive peptides are indicated by a broken line. The asterisk marks the cDNA's stop codon.

METHODS. Degenerate oligonucleotides were designed on the basis of the amino acid sequence of the new peptide, taking into account the frequency of codon usage in mammals. Direct primer was AGATCTA-GACTT(CT)GG(CG)GG(CG)TT(CT)AC(ACGT)GG and reverse primer was CTTAAGCTT(CT)TG(AG)TT(ACGT)GC(CG)AG(CT)TT, corresponding to sequences 1–6 and 13–17 of the peptide, respectively. These oligonucleotides were used to amplify and clone by PCR on human, rat and mouse genomic DNA, the gene fragment encoding the peptide. The deduced central amino acids of the peptide (ARKSAR) were identical in the three species. The rat clone was used as a probe to screen a rat-brain cDNA library (Stratagen) constructed in lambdaZAPII. A single cDNA clone was isolated and sequenced.

precursor whose mRNA is expressed in the brain. The new peptide, whose sequence appears to be strictly conserved across mammalian species (ox (not shown), mouse, rat and man) (see legend to figure 4), must be of widespread importance. Because its first demonstrated physiological action appears to be increased reactivity to pain in intact animals, the new peptide might be named nociceptin. □

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# Negative regulation of T-cell adhesion and activation by CD43

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**CD43 is a cell-surface sialoglycoprotein expressed by a variety of haematopoietically derived cells, including T lymphocytes[1–9]. Earlier observations of defective CD43 expression by T lymphocytes from boys with the X-chromosome-linked Wiskott–Aldrich syndrome suggested the importance of CD43 in lymphocyte function[10,11]. Subsequent studies have suggested that CD43 facilitates leukocyte adhesion[12–14] and has a co-stimulatory role during T-cell activation[15]. To define the physiologically relevant function(s) of CD43, we have generated CD43-knockout mice. We report here that CD43-deficient T cells from such mice show a marked increase in their *in vitro* proliferative response to concanavalin A, anti-CD3, the superantigen SEB and allostimulation. Additionally, CD43-deficient T cells show a substantial enhancement in homotypic adhesion and in their ability to bind different ligands, including fibronectin and the intercellular adhesion molecule ICAM-1. Vaccinia-virus-infected CD43-knockout mice mounted an augmented anti-vaccinia cytotoxic T-cell response compared with their wild-type littermates, yet developed an increased virus load. We conclude that CD43 negatively regulates T-cell activation and adhesion and is important for viral clearance.**

CD43-knockout mice were generated by homologous recombination via embryonic stem-cell chimaeras (Fig. 1). Although CD43 is expressed from a very early stage in haematopoiesis[8,9] and the pattern of expression varies in a lineage-specific manner[16–19], no developmental defect was observed in the mutant mice. The mice develop and breed normally. Haematocrit values were normal, as were blood leukocyte and platelet counts. Histology of bone marrow, thymus, spleen and lymph nodes from CD43-knockout mice resembled that of wild-type mice. Flow-cytometric analysis of thymocytes and splenocytes revealed no gross immunophenotypic abnormalities other than the loss of CD43 expression. T-lymphocyte numbers, CD4 and CD8 subset proportions, levels of CD3, CD4 and CD8 expression, and B-cell ($B220^+$, $IgM^+$) numbers in spleens from CD43-knockout mice were comparable to those of their wild-type littermates. T-lymphocyte expression of activation markers, including interleukin-2α chain, Ia antigen and lymphocyte function-associated antigen-1 (LFA-1), were also comparable. Surface-antigen expression on marrow cells from the knockout mice was similar to that of wild-type mice, with the exception that most $B220^+$ cells in knockout marrow were $Mac\text{-}1^+$, a finding of uncertain functional relevance. We conclude that CD43 is not essential for haematopoiesis.

Previous studies have shown that monoclonal antibodies to human CD43 can be co-stimulatory for T cells[12] or activate them directly[20]. More recently, expression of human CD43 by a murine T-cell hybridoma was observed to enhance its response to antigen[15]. We therefore tested T cells from the CD43-knockout mice and their wild-type littermates for their ability to proliferate in response to T-cell activators. Splenocytes were stimulated with the T-cell mitogen concanavalin A (conA), the anti-CD3 monoclonal antibody 145-2C11, or the superantigen staphylococcal enterotoxin B (SEB) at various concentrations. Thymocytes were stimulated with anti-CD3 and phorbol-12-myristyl-13-acetate (PMA). In each case, T cells from knockout mice showed substantially enhanced proliferation compared with those from their wild-type littermates (Fig. 2*a*). Moreover, enhanced proliferation was evident at all concentrations of stimulants tested, although maximal enhancement was seen with suboptimal concentrations of stimulants (not shown). To assess the dynamics of proliferation of $CD43^+$ and $CD43^-$ T cells, different cell numbers were tested for proliferation at different time points using various concentrations of conA. At 48 h, the enhancement in proliferation of the CD43-deficient T cells was most striking when either conA concentration or cell number was limiting (Fig. 2*b*). By contrast, under optimal stimulatory conditions ($2 \times 10^5$ cells, 2 μg $ml^{-1}$ conA), only a modest increase (50%) in $^3H$-thymidine incorporation by the CD43-deficient cells was observed. However, at 24 h, duplicate cultures under identical stimulatory conditions showed a 300% increase in $^3H$-thymidine incorporation by the CD43-deficient cells (not shown). These results suggest an accelerated kinetics of T-cell activation in the absence of CD43.

To determine whether the proliferation of CD43-deficient T cells was also enhanced in response to a natural antigen, alloreactivity of T cells was tested. Again, proliferation of T cells from the CD43-knockout mice was more than double that of cells from their wild-type counterparts ($23.1 \pm 1.8 \times 10^3$ c.p.m. compared with $9.4 \pm 0.7 \times 10^3$ c.p.m., respectively). A similar increase in CD43-deficient T-cell proliferation was also seen after secondary and tertiary stimulation (not shown), indicating an increase in the proliferative ability of the CD43-deficient T cells rather than in the number of lymphocytes in the allospecific precursor pool.

To investigate possible *in vivo* consequences of CD43-deficiency, cytotoxic T-cell response to vaccinia virus infection in the knockout mice was compared with that in their wild-type littermates. Seven days after infection with a high virus dose ($10^6$ $TCID_{50}$ (half-maximal tissue culture infective dose)), comparable levels of anti-vaccinia cytotoxic T-lymphocyte (CTL) activity were found in both mouse groups (not shown). By contrast, when limiting amounts of virus ($2 \times 10^3$ $TCID_{50}$) were used, a significantly greater (fourfold) CTL response was consistently observed in the CD43-deficient mice (Fig. 3*a*). These