Biological Activity of Recombinant Human Interleukin-2 Produced in *Escherichia coli*

Abstract. *The gene for interleukin-2 was isolated from the Jurkat cell line and from normal peripheral blood lymphocytes and, when inserted in* Escherichia coli, *was expressed at high concentrations. This interleukin-2 was purified to apparent homogeneity and tested for biological activity in a variety of assays in vitro and in vivo. The recombinant lymphokine supports the growth of murine and human interleukin-2 dependent cell lines, enhances the generation of murine and human cytolytic cells in vitro, and generates lymphokine activated killer cells from murine and human lymphocytes. It has a serum half-life of 2 to 3 minutes in the mouse and significantly enhances the generation of cytolytic cells in vivo after alloimmunization. No functional differences between native and the recombinant interleukin-2 molecules have been detected.*

Human interleukin-2 (IL-2), a glycoprotein with a molecular weight of approximately 15,000, is produced by human lymphocytes that have been stimulated by mitogens or antigens. Although originally described as a factor capable of maintaining long-term growth of T cells in culture, IL-2 has been shown to mediate a large number of immunologic phenomena in vitro and in vivo and appears to play a central role in the generation of immune responses (*1*).

In vitro, cell culture supernatants containing IL-2 act as helper factors in B- and T-cell responses, augment the generation of cell-mediated cytotoxic T lymphocytes, maintain long-term proliferative and cytotoxic cell cultures, stimulate lymphokine-activated killer cell activity, and mediate the recovery of the immune function of lymphocytes in selected immunodeficient states (*2*). The administration of IL-2 in vivo has been shown to enhance natural killer function, augment alloantigen responsiveness, improve the recovery of immune function in acquired immunodeficient states, induce immune functions in nude mice, and mediate antitumor effects when administered in conjunction with immune lymphocytes (*3*).

Human IL-2 is produced in minute quantities by the helper T cells present in peripheral blood lymphocytes (PBL). Although high-producer tumor lines such as the Jurkat leukemia cell line, the Gibbon MLA 144 line, or T-cell hybridomas produce increased levels of IL-2, the limited amount of purified native IL-2 obtainable from these sources has been a major impediment to studies of the biological role of this lymphokine (*4*).

Taniguchi *et al.* isolated a human IL-2 complementary DNA (cDNA) clone from the Jurkat cell line and determined its nucleotide sequence (*5*). This cDNA was expressed in transfected cultured monkey cells, and a biologically active polypeptide was produced. In similar experiments, Devos *et al.* isolated a cDNA clone coding for IL-2 from lymphocytes and succeeded in causing this gene to be expressed in *Escherichia coli* (*6*). A 15,000-dalton polypeptide with IL-2 activity was detected on sodium dodecyl sulfate (SDS) gels of the total *E. coli* lysate. The use of recombinant DNA techniques to produce large amounts of IL-2 in *E. coli* is expected to greatly facilitate biological and clinical studies of this molecule.

Here we describe the cDNA cloning of the gene for IL-2 from the Jurkat cell line and from normal human PBL. Nucleotide sequence analysis of the IL-2 clones from both of these sources revealed that they are identical except for minor base changes and that they encode identical mature IL-2 proteins. Therefore, only one of the Jurkat cDNA clones has been engineered for expression in *E. coli*. Large amounts of IL-2 have been produced by using this construct, and the protein has been purified to apparent homogeneity. The IL-2 from this source has the in vitro and in vivo biological activities of native IL-2 produced directly by human lymphocytes.

The human Jurkat cell line was subcloned to generate a high IL-2 producer line, Jurkat-A5. The Jurkat-A5 line (2×10^6 cells per milliliter) was induced for production of IL-2 by treatment with phorbol myristate acetate (10 ng/ml) and phytohemagglutinin (3 μg/ml) for 8 hours. Human PBL were purified by Ficoll-Hypaque centrifugation and induced for IL-2 production (4×10^6 cells per milliliter) with phorbol myristate acetate (5 ng/ml) and concanavalin A (10 μg/ml) for 48 hours. Total cytoplasmic RNA's were extracted (*7*) from both types of cells, and polyadenylate [poly(A)]-containing RNA [messenger RNA (mRNA)] was purified by oligo-deoxyri-

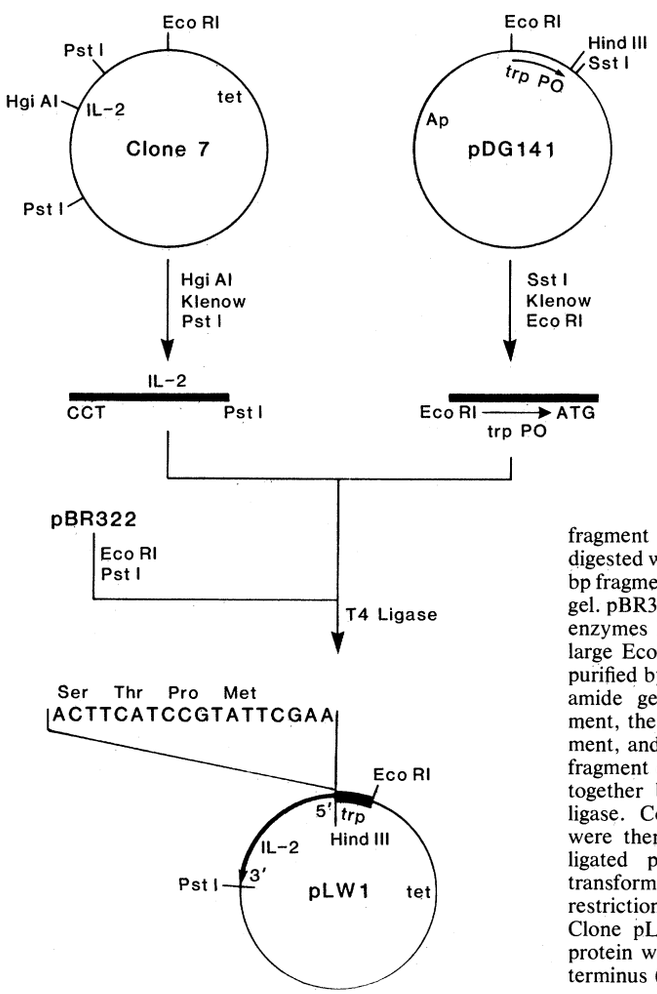

Fig. 1. Cloning of the gene for IL-2. Clone 7 DNA was digested with Hgi Al and the 3′-protruding end removed by treatment with DNA polymerase I Klenow fragment. The blunt-ended DNA was then digested with Pst I and the 706-bp fragment containing the coding sequence of IL-2 was purified. The *trp* promoter fragment, containing an ATG initiation codon, was purified from pDG141 by digestion with Sst I, treated with DNA polymerase I Klenow fragment to create a blunt end, digested with Eco RI, and the 120-bp fragment was recovered from a gel. pBR322 was digested with the enzymes Eco RI and Pst I. The large Eco RI–Pst I fragment was purified by fractionation on acrylamide gel. The promoter fragment, the 706-bp IL-2 DNA fragment, and the large Eco RI–Pst I fragment of pBR322 were ligated together by means of T4 DNA ligase. Competent *E. coli* cells were then transformed with the ligated plasmid. The resulting transformants were checked by restriction enzyme mapping. Clone pLW1 codes for an IL-2 protein with Met-Pro at its NH_2-terminus (des-alanyl-IL-2).

bosylthymine-cellulose chromatography (*8*). The Jurkat and PBL mRNA's (250 μg of each) were fractionated on gradients of 5 to 20 percent sucrose. Portions from each fraction were tested for IL-2 mRNA activity by injection into frog oocytes (*9*) and by subsequent testing of the oocyte incubation medium for IL-2 biological activity on a murine IL-2 dependent cell line (*10*). The fractions containing the peak IL-2 mRNA activity were pooled and used for construction of cDNA banks.

Single-stranded cDNA's were synthesized in the presence of 2 m*M* vanadyl-adenosine complex (*7*), and the second-strand cDNA's were synthesized according to the oligonucleotide priming method described by Rougeon *et al.* (*11*). The cDNA's from both the human PBL and the Jurkat cell line were inserted into the Pst I site of pBR322 by the G-C (guanine-cytosine) tailing method (*12*) and were transformed into *E. coli* K-12 strain MM294 to generate two cDNA banks.

The IL-2 cDNA clones in the Jurkat cDNA bank were identified by filter-hybridization (*13*). A ^{32}P-labeled 20-mer oligonucleotide probe with the sequence GTGGCCTTCTTGGGCATGTA (T, thymine; A, adenine), complementary to the region near the middle of the IL-2 gene [see (*5*)], was chemically synthesized and used in screening the cDNA bank. With the use of this synthetic probe, 21 clones were hybridized out of 10,000 clones tested; from these, six full-length clones were identified and confirmed by restriction enzyme mapping. The cDNA inserts from two of the full-length clones (clones 7 and 8) were completely sequenced. Clone 8 had the same DNA sequence as that of the IL-2 clone described by Taniguchi *et al.* (*5*) and clone 7 differed by having one more base (T) at the 5′ end and two other single base changes at position 77 (T → C) and at position 504 (A → G). These changes did not affect the amino acid sequence of the mature protein.

Ten thousand clones from the PBL cDNA library were screened by colony hybridization with the 490-base pair Rsa I/Stu I coding sequence fragment from Jurkat IL-2 clone 7. Twenty-four positive clones were identified, and nine of these clones were found to have restriction maps consistent with IL-2. The inserts from two of the full-length clones (clone 26 and clone 60) were sequenced. Clone 60 was identical to the Jurkat clone 8, and clone 26 had a single base change at position 161 (G → A).

The *E. coli trp* promoter has been used extensively by us (*14*) and others (*15*) for the efficient expression of cloned genes in *E. coli*. We constructed the plasmid pLW1 for the expression of human IL-2 under the control of the *E. coli trp* promoter using the scheme described in Fig. 1. As a result of the construction protocol, this plasmid encodes an IL-2 protein which has a methionine replacing the NH_2-terminal alanine found in native IL-2. We also constructed a plasmid that encodes an IL-2 protein with methionine-alanine at its NH_2 terminus. The IL-2 proteins purified from both of these constructs appear to have biological activities similar to those of native IL-2.

When the *E. coli* cell harboring pLW1 was grown in the absence of tryptophan to induce the *trp* promoter, and total cell extracts analyzed on SDS-polyacrylamide gels, a new protein of approximately 15,000 daltons was induced (lane 3 in Fig. 2). The induced IL-2 represents about 5 percent of the total cellular protein based on densitometric scans of Coomassie-stained SDS gels. When this extract was assayed for IL-2 activity on an IL-2 dependent mouse cell line, it was found to contain about 10^5 units of IL-2 activity per milliliter of bacterial culture. Before we tested the biological activity of recombinant IL-2 in vivo and in vitro, milligram amounts of the protein were purified to apparent homogeneity (lane 4 in Fig. 2). The endotoxin level in the purified preparation was routinely less than 0.1 ng per 10^5 units of IL-2, as measured in a standard Limulus assay. Various preparations of IL-2 with similar purity were used in all subsequent experiments.

The recombinant human IL-2 was tested for biological activity in a variety of

Table 1. Effect of recombinant IL-2 in allosensitization cultures with nonimmunogenic stimuli. The results are expressed as lytic units per 10^6 cells. N.D., not done.

IL-2 (unit/ml)	Stimulator cells: X-irradiated (3300 rads)	Heat-killed	Ultraviolet-irradiated
	*Mouse splenocytes**		
0	6	N.D.	< 1
32	5.4	N.D.	4.2
160	25.0	N.D.	25.0
800	30.3	N.D.	37.0
4,000	22.2	N.D.	41.7
20,000	7.4	N.D.	15.2
100,000	5.3	N.D.	5.6
	Human PBL†		
0	0.25	< 0.1	1.4
100	6.3	6.3	7.4

*C57BL/6 splenocytes (4×10^6) were incubated with 10^6 DBA/2 stimulator splenocytes in 2 ml of complete medium in 24-well culture plates. Complete medium was composed of RPMI 1640 containing 10 percent fetal calf serum, 0.03 percent glutamine, 1 m*M* sodium pyruvate, 0.1 m*M* nonessential amino acids, 5×10^{-5} *M* 2-mercaptoethanol, penicillin (100 unit/ml), and streptomycin (100 μg/ml). After 4 days the cultures were harvested and tested in a 4-hour ^{51}Cr-release assay for lysis of DBA/2 target cells at a variety of effector-to-target cell ratios. One lytic unit was defined as the number of effector cells required to cause 50 percent lysis of 10^4 target cells (or in the human assays, 5×10^3 target cells). No lysis of C57BL/6 target cells was seen in any assay. †Fresh responder PBL (10^7) were incubated with 10^7 stimulator allogeneic PBL in 10 ml of medium in Falcon 3013 flasks incubated vertically. Medium consisted of RPMI 1640 with 10 percent human AB serum, penicillin (100 unit/ml), and streptomycin (100 μg/ml). After 7 days, flasks were harvested and tested for lysis against ^{51}Cr-labeled stimulator lymphocytes. No lysis was seen against ^{51}Cr-labeled responder lymphocytes.

Table 2. Generation of lymphokine-activated killer (LAK) cells by recombinant IL-2. The results are expressed as lytic units per 10^6 cells. N.D., not done.

Concentration of IL-2 (unit/ml)	Generation of LAK cells: Mouse*	Human†
1	< 1	< 1
6	< 1	1.0
32	< 1	4.4
160	< 1	6.3
800	10.0	11.8
4,000	14.7	66.6
20,000	5.9	22.7
100,000	2.7	N.D.

*Murine LAK cells were generated by incubating 4×10^6 splenocytes in 2 ml of medium in 24-well culture plates containing various concentrations of recombinant IL-2 for 5 days. Lysis of fresh syngeneic MCA-102 sarcoma cells was tested in a 4-hour ^{51}Cr-release assay. MCA-102 is an NK-resistant methylcholanthrene induced sarcoma used in the first six transplant generations. Single cell suspensions were obtained as previously described (*20*). †Human LAK cells were generated by incubation of 2×10^6 fresh PBL per well for 4 days. Lysis was tested with fresh uncultured target cells obtained from a human sarcoma.

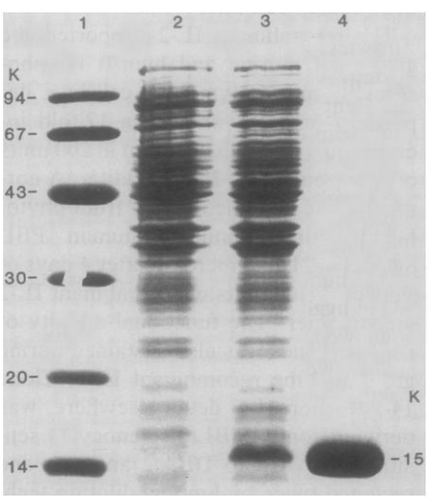

Fig. 2. Polyacrylamide gel electrophoresis of IL-2 produced by *E. coli*. The *E. coli* containing recombinant IL-2 clone pLW1 was grown in minimal medium in the absence of tryptophan to derepress the *trp* promoter, and total cell extracts were analyzed on a 15 percent SDS-polyacrylamide mini-gel stained with Coomassie blue. A new protein of about 15,000 daltons is seen in extracts from pLW1 (lane 3) which is not present in extracts of *E. coli* containing the plasmid vector without the IL-2 gene (lane 2). The IL-2 protein produced in *E. coli* has been purified to apparent homogeneity and approximately 5 μg of this purified IL-2 was analyzed in lane 4.

Table 3. Enhancement of the generation of cytolytic cells with recombinant IL-2.

Mouse	Challenge with $P815_x$	IL-2 (unit)	Specific lysis of P815*	
			100:1	50:1
Immune†	Yes	0	10 ± 1	8 ± 1
	Yes	100	16 ± 1	10 ± 1
	Yes	1,000	21 ± 1	15 ± 2
	Yes	10,000	45 ± 3	30 ± 3
Immune†	No	0	0 ± 1	0 ± 1
	No	100	1 ± 2	1 ± 1
	No	1,000	−1 ± 1	4 ± 2
	No	10,000	3 ± 1	3 ± 2
Normal‡	No	0	1 ± 1	1 ± 1
	No	100	7 ± 4	1 ± 1
	No	1,000	−1 ± 1	7 ± 5
	No	10,000	3 ± 2	6 ± 1

*Percentage lysis ± standard error of the mean. †Irradiated (10,000 rads) P815 cells (10^7) were injected intraperitoneally into allogeneic C57BL/6 mice. Fourteen days later one-half of the mice were challenged with 10^6 irradiated P815 cells injected intraperitoneally. On days 15, 16, and 17 the mice received either Hanks basic salt solution (HBSS) or recombinant IL-2, at the concentration indicated, in 0.5 ml, three times a day. On day 21, spleens from these mice were assayed for lysis of P815 target cells in a 4-hour ^{51}Cr-release assay intraperitoneally. ‡Normal nonimmune C57BL/6 mice received HBSS or recombinant IL-2 by the schedule outlined above.

immunologic assays with murine and human cells. The IL-2 was highly active in standard assays of IL-2, as determined by the ability of test preparations to sustain [^{3}H]thymidine incorporation into cell lines dependent on naturally derived IL-2 (*10*). For the studies reported here, the titer, in units per milliliter, is defined as the reciprocal of the dilution required to sustain one-half of the maximum [^{3}H]thymidine incorporation into 5×10^3 cells from a long-term IL-2 dependent murine line grown in our laboratory (line 53). Similar titers were obtained when samples were tested on an IL-2 dependent human cell line.

The recombinant IL-2 supported the growth of murine and human lymphocytes. A murine lymphoid cell line, dependent on IL-2, showed a 17-fold increase in 6 days when grown in 200 units of recombinant IL-2 per milliliter. A normal human cell line derived from phytohemagglutinin-stimulated human PBL showed a 21-fold increase after 4 days of growth in 100 units of recombinant IL-2 per milliliter. The functional activity of murine clones was also sustained during growth in the recombinant IL-2. Clone 14-11, reported in detail elsewhere, was derived from C57BL/6 splenocytes sensitized in vitro to DBA/2 antigens and recloned twice by limiting dilution techniques (*16*). This clone exhibited a high degree of specific proliferation in response to H-2^d antigens and maintained this reactivity when expanded over 10^6-fold during 6 weeks of growth in recombinant IL-2.

The ability of recombinant IL-2 to provide helper activity in the generation of cytolytic cells is shown in Table 1. In the experiment with murine cells, the responder lymphocytes were incubated with x-irradiated or ultraviolet (UV)-irradiated stimulator splenocytes for 4 days in various concentrations of recombinant IL-2. In cultures containing x-irradiated stimulator cells, 160 units of IL-2 per milliliter enhanced the generation of specific alloreactive cells by 4.2-fold while 800 unit/ml provided an increase of 5.1-fold. The recombinant IL-2 also stimulated the generation of specific cytotoxic cells when nonimmunogenic UV-irradiated stimulator cells were used. In the absence of IL-2, less than 1 lytic unit was generated compared to 37 lytic units when 800 units of recombinant IL-2 were present. Table 1 also shows that the recombinant IL-2 could enhance the generation of human allocytotoxic cells when x-irradiated, heat-killed, or UV-irradiated stimulated PBL were used. No lytic activity against stimulator PBL was generated after culture of responder PBL alone in 100 units of IL-2 per milliliter. Thus, the recombinant IL-2 is capable of totally replacing the proliferative signal required for sensitization to alloantigens in vitro. This is a fundamental property of IL-2 derived from natural sources (*17*).

The recombinant IL-2 was also effective in generating lymphokine-activated killer cells from resting murine splenocytes and human PBL. Various concentrations of the recombinant IL-2 were added to murine splenocytes or fresh human PBL and incubated for 4 to 5 days. These cells were then tested in a 4-hour ^{51}Cr-release assay for the ability to lyse fresh, uncultured, natural killer (NK)-resistant tumor targets obtained from mouse or human tumors by enzymatic digestion. Table 2 shows that 4000 units of IL-2 per milliliter could generate highly effective lymphokine-activated killer cells in both the mouse and the human. Normal lymphocytes were not lysed by these killer cells.

Measurement of circulating concentrations of IL-2 in mice injected intravenously with the recombinant lymphokine indicated a serum half-life of 2 to 3 minutes. This short half-life in vivo is similar to the half-life of natural murine IL-2 injected intravenously in the mouse (*18*). We also found that 10,000 units of the recombinant IL-2 administered intraperitoneally three times a day on the first, second, and third days after a secondary challenge of C57BL/6 mice with allogeneic cells significantly increased the generation of specific allocytotoxic cells in vivo (Table 3). These results are similar to those we previously reported with naturally derived IL-2 in this model system (*10*).

The ability of the purified recombinant IL-2 to replace crude, IL-2-containing, lymphokine preparations in these various biological systems confirms that IL-2 is the active ingredient. There is now considerable interest in the role of lymphokine-activated killer cells in the immunotherapy of murine tumors and the possibility of using this approach in cancer treatment (*19*). The availability of large amounts of recombinant IL-2 will make it feasible to generate lymphocyte activated killer cells for use in clinical trials of this approach in humans and will facilitate studies of the biological role of IL-2 in immunologic processes.

STEVEN A. ROSENBERG
ELIZABETH A. GRIMM
Surgery Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205
MICHAEL MCGROGAN
MICHAEL DOYLE
ERNEST KAWASAKI
KIRSTON KOTHS
DAVID F. MARK
Cetus Corporation, Emeryville, California 94608

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Increased Phosphorylation of Myosin Light Chain Kinase After an Increase in Cyclic AMP in Intact Smooth Muscle

Abstract. *The role of cyclic adenosine monophosphate–mediated phosphorylation of myosin light chain kinase in relaxing smooth muscle was examined. The kinase was immunoprecipitated from tissue extracts and the phosphate content was determined. The addition of forskolin to resting or methacholine-contracted muscles resulted in an increase in myosin light chain kinase phosphorylation and a relaxation of contracted muscles. These findings suggest that phosphorylation of myosin light chain kinase is one of the reactions in the process by which cyclic adenosine monophosphate causes relaxation of smooth muscle.*

Myosin phosphorylation is essential for smooth muscle contraction (*1–3*). The mechanisms that regulate smooth muscle relaxation, however, are not known. In recent in vitro experiments phosphorylation by adenosine 3′,5′-monophosphate (cyclic AMP)–dependent protein kinase decreased the catalytic activity of myosin light chain kinase (MLCK) (*4*). Since β-adrenergic receptor stimulation increases cyclic AMP and causes relaxation of smooth muscles (*5*), it has been suggested that a decrease in MLCK activity after phosphorylation and the subsequent dephosphorylation of myosin by phosphatases (*1, 2*) could be part of the mechanism of cyclic AMP–mediated relaxation. However, indirect experiments on the state of MLCK phosphorylation in intact smooth muscle have provided contradictory results (*6*). Therefore we performed experiments to correlate MLCK phosphate content with cyclic AMP levels, myosin phosphate content, and tension in intact canine tracheal smooth muscle treated with pharmacological agents.

Resting (noncontracted) tracheal muscles incubated with ^{32}P (Fig. 1) were frozen after being treated with forskolin, an agent that increases cyclic AMP (*7*) and relaxes smooth muscles (Table 1). The frozen muscles were homogenized in boiling sodium dodecyl sulfate (SDS) and MLCK was immunoprecipitated from the SDS extracts with affinity-purified antibodies to turkey gizzard MLCK (*8*). Control experiments have demonstrated that the phosphate content of MLCK does not change during purification. Polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1A) showed a single protein band with a molecular weight of about 155,000 in the immunoprecipitates. This value is in good agreement with that reported for MLCK purified from bovine stomach muscle (*9*). An autoradiograph of this gel (Fig. 1B) demonstrated that MLCK was phosphorylated in resting muscle and that phosphorylation increased on treatment with forskolin. In addition, it appeared that MLCK phosphorylation increased within 1 or 2 minutes after forskolin was added.

Experiments on the stoichiometry of phosphorylation were performed on tracheal muscles incubated with ^{33}P. The muscles were treated with various pharmacological agents and frozen (Table 1). MLCK was immunoprecipitated from half of each muscle (see legend to Fig. 1) and the extent of MLCK phosphorylation was determined (see legend to Table 1). ATP was purified from the other half of each muscle with affinity chromatography and high-performance liquid chromatography and the specific activity was determined. Control experiments demonstrated that the ^{33}P was in isotopic equilibrium with intracellular ATP within 60 minutes, that more than 90 percent of the purified ATP was labeled in the gamma position, and that the ATP was free of other nucleotides (*10*). The MLCK phosphorylation data were then compared with the data on cyclic AMP, myosin phosphate (PO_4), and relaxation.

Addition of forskolin to resting muscles (Table 1) caused significant increases in cyclic AMP (from 4.4 to 71.0 pmole per milligram of protein) and MLCK PO_4 (from 1.0 to 1.7 moles per mole of MLCK) and a slight but statistically significant decrease in myosin phosphorylation. Methacholine did not change the cyclic AMP content or the MLCK PO_4 content but did increase myosin phosphorylation and tension (*2*). Addition of atropine to methacholine-contracted muscles decreased both the myosin PO_4 content and tension to the resting levels. Addition of forskolin to methacholine-contracted muscles was accompanied by a significant decrease in myosin phosphate content and tension, although neither value returned to the resting level. Moreover, cyclic AMP was elevated and MLCK phosphorylation was near the maximum level of 2 moles of PO_4 per mole of MLCK when methacholine-contracted muscles were relaxed with forskolin.

The level of phosphorylation in resting tracheal muscle is surprising. We had anticipated a lower level of phosphorylation than the 1 mole of PO_4 per mole of MLCK measured in resting, untreated tracheal muscles. Torphy *et al.* (*11*) recently reported that as much as 24 percent of the protein kinase molecules activatable by cyclic AMP are active in resting canine tracheal smooth muscle. This level of active cyclic AMP–dependent protein kinase may be sufficient to maintain 50 percent of the available MLCK sites in the phosphorylated state.

The identity of the site on MLCK that is phosphorylated in resting tracheal smooth muscle is of interest. It is possible to phosphorylate two sites on turkey gizzard smooth muscle MLCK, and both sites apparently must be phosphorylated before there is a reduction in MLCK catalytic activity (*4*). Thus phosphorylation would be most effective in modulating MLCK activity if one site is phosphorylated on all MLCK molecules in resting muscle and both sites become phosphorylated after the addition of forskolin.

The presence of 1.7 ± 0.1 moles of PO_4 per mole of MLCK in resting muscle treated with forskolin suggests that a significant fraction of the MLCK molecules are phosphorylated at two sites.