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Inhibition by Serotonin of Amino Acid Release and Protein Degradation in Skeletal Muscle

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SUMMARY

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The effect of serotonin (5-hydroxytryptamine) on muscle amino acid and protein metabolism was investigated *in vitro*, using the isolated intact epitrochlaris preparation of rat skeletal muscle. Increasing concentrations of serotonin (1 nM–0.1 mM) produced a concentration-dependent decrease in the rate of alanine and glutamine release from muscle; intracellular levels of these amino acids were also decreased slightly by serotonin. Muscle alanine reutilization, as determined by [*U*-^{14}C]alanine oxidation to $^{14}CO_2$ and incorporation into protein, was unaffected by serotonin, and maximal rates of muscle protein synthesis, as judged by [^{3}H]leucine incorporation into trichloroacetic acid-precipitable protein, were also unaltered. These effects of serotonin were blocked by cyproheptadine and methysergide, but were unaffected by morphine and tetrodotoxin (0.1 μg/ml). None of these antagonists exhibited substantial independent effects on muscle alanine and glutamine synthesis and release. Exogenous serotonin resulted in a concentration-dependent increase in steady-state levels of intracellular cyclic 3′,5′-AMP in these muscle preparations. Theophylline (1 mM) or isobutylmethylxanthine (0.2 mM) potentiated the effects of submaximal concentrations of serotonin (1 μM) on muscle amino acid release, and amplified the increments in muscle cyclic AMP produced by this concentration of serotonin. Cyproheptadine and methysergide, but not morphine or tetrodotoxin, blocked the serotonin-induced increments in muscle cyclic AMP levels. The effects of serotonin on alanine and glutamine synthesis and release from muscle were reproduced by exogenous dibutyryl cyclic AMP. Serotonin did not reduce the rate of formation of alanine and glutamine from exogenous amino acid precursors such as aspartate, cysteine, leucine, and valine. Skeletal muscle proteins in the epitrochlaris preparations were partially labeled with [*guanido*-^{14}C]arginine administered *in vivo* using pulse-chase methods. On incubation of these preparations *in vitro*, serotonin decreased the rate of loss of ^{14}C label from trichloroacetic acid-precipitable protein in the muscles. Correspondingly, serotonin also decreased the appearance of ^{14}C label in the incubation medium and in the supernatant fraction of the muscles obtained after trichloroacetic acid treatment. These data indicate that serotonin inhibits alanine and glutamine synthesis and release from intact skeletal muscle. This effect is mediated by a specific D-class serotonin receptor and appears to require the intermediary participation of muscle cyclic AMP. The mechanism of serotonin action most probably involves

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primary inhibition of protein degradation, which thereby results in decreased availability of precursor amino acid substrates for conversion to alanine and glutamine. These results indicate that serotonin, like norepinephrine, has direct effects on skeletal muscle metabolism, and that these effects are independent of its vasoactive properties.

INTRODUCTION

In previous studies on skeletal muscle metabolism using the isolated intact epitrochlaris preparation of rat skeletal muscle, we demonstrated that epinephrine and norepinephrine had direct effects on skeletal muscle amino acid metabolism and release (1). We have shown also that alanine and glutamine are preferentially synthesized in skeletal muscle from a variety of other intracellular amino acids (2, 3). As a consequence of this selective synthesis, rates of alanine and glutamine release from skeletal muscle account for approximately two-thirds of the total amount of amino acids released from muscle (2). We also observed that the rate of alanine and glutamine formation and release appeared to be closely associated with the rate of protein degradation in muscle, and not with glycolytic processes (4). It is skeletal muscle protein degradation, therefore, which must maintain the continued availability of intracellular precursor amino acids for conversion to alanine and glutamine. Norepinephrine and epinephrine, acting by way of a *beta* adrenergic receptor and variations in intracellular cyclic 3',5'-AMP, inhibited skeletal muscle alanine and glutamine formation and release (1). These observations indicate that catecholamines may exert significant regulatory actions on protein and amino acid balance in skeletal muscle. The present study was undertaken to investigate the effects of serotonin (5-hydroxytryptamine), another autacoid, on muscle cAMP[2] metabolism, on the synthesis and release of alanine and glutamine, and on rates of protein degradation in the isolated intact epitrochlaris preparation of rat skeletal muscle.

MATERIALS AND METHODS

Adenylate kinase (EC 2.7.4.3), alanine aminotransferase (EC 2.6.1.2), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glutamate dehydrogenase (EC 1.4.1.3), glutaminase (EC 2.5.1.2), hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), NAD^+, NADH, $NADP^+$, serotonin, and tetrodotoxin were obtained from Sigma Chemical Company. Radioisotopes were purchased from New England Nuclear. Cyproheptadine HCl was a gift of Merck Sharp & Dohme Research Laboratories. Methysergide maleate was kindly supplied by Sandoz Pharmaceuticals.

Male Sprague-Dawley rats weighing 125–150 g were killed by a blow to the high cervical spine, and epitrochlaris preparations were visualized and rapidly removed intact as previously described (2). Excess connective tissue was trimmed, and the muscle was rinsed briefly in isotonic sodium chloride, blotted, and placed in a modified Krebs-Henseleit buffer (pH 7.4) containing one-half the calcium concentration as originally formulated, and with increased buffering capacity obtained by the addition of 5 mM HEPES (pH 7.4). Other additions to the medium, such as glucose (5 mM) or serotonin, were made as indicated. The incubation flasks were gassed with 95% O_2–5% CO_2 and stoppered, and incubations were carried out in a shaking water bath at 37° for 30–360 min. At the end of the incubation, the muscles were removed rapidly, rinsed in cold buffer, blotted, and frozen in liquid nitrogen. All muscle samples and incubation media were stored at −80°. Frozen muscle tissue was weighed while maintained at −20°, then rapidly homogenized in 3 M perchloric acid and subsequently centrifuged at 4500 × *g* for 15 min. The resulting supernatant was neutralized with a solution containing 3 M potassium hydroxide, 0.5 M imidazole, and 0.7 potassium chloride, and the potassium perchlorate precipitate was removed by further centrifugation (5).

For studies of protein synthesis *in vitro*, experiments were carried out in incuba-

[2] The abbreviations used are: cAMP, adenosine cyclic 3',5'-monophosphate; cGMP, guanosine cyclic 3',5'-monophosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

tion flasks under continuous gassing with 95% O_2–5% CO_2 for 2–4 hr. The incubation medium was further modified by the addition of 17 essential and nonessential amino acids (2.5 mM each) and insulin (100 munits/ml). [4,5-^{3}H]Leucine (1.0 μCi/ml) was also added. Following incubation, isotopic amino acid incorporation into trichloracetic acid-precipitable protein was determined by rinsing, homogenization of the muscle in 12% trichloracetic acid, and subsequent solubilization of the washed, pelleted precipitate with NCS solubilizer and liquid scintillation counting using Aquasol. Studies of alanine oxidation by intact epitrochlaris preparations were carried out similarly, except that alanine was the only added amino acid (final concentration, 0.4 mM), and [*U*-^{14}C]alanine was included at a final concentration of 1 μCi/ml. The evolution of $^{14}CO_2$ was determined by acidification of the incubation medium with 2 N H_2SO_4 and absorption of the $^{14}CO_2$ evolved within the flask into a well containing triethanolamine. The latter was then transferred to liquid scintillation vials for counting. Alanine specific activities were determined from aliquots of the incubation medium prior to and after incubation. The incorporation of alanine into trichloracetic acid-precipitable protein of epitrochlaris preparations was determined using an incubation medium similar to that used for studies of protein synthesis, except that [*U*-^{14}C]alanine was the radioisotope added (0.4 mM; 1.0 μCi/ml). Alanine specific activities were determined before and after incubation. Radiolabeling of trichloracetic acid-precipitable protein was determined as outlined above.

For studies of protein degradation *in vitro*, epitrochlaris muscles were previously labeled *in vivo*, using an initial intraperitoneal injection of 50 μCi of [*guanido*-^{14}C]arginine (specific activity, 55 mCi/mmole) followed 6 hr later by a second intraperitoneal injection of unlabeled arginine (20 μmoles). Twenty-four hours later the animals were killed and epitrochlaris preparations were obtained and incubated for 2–4 hr in 0.5 ml of Krebs-Henseleit buffer. Additions to the medium were made as indicated. Following incubation, muscles were rinsed, blotted, and immediately frozen in liquid nitrogen. The tissues were subsequently homogenized in 0.9 ml of trichloracetic acid (12%) and centrifuged for 20 min at 2500 × *g*. The post-trichloracetic acid supernatant and the incubation medium were directly sampled for liquid scintillation counting. The precipitate itself was washed twice with additional trichloracetic acid and subsequently with diethyl ether, with final drying in air. This precipitate was dissolved in 0.2 ml of NCS solubilizer, transferred to liquid scintillation vials, and counted using ACS scintillant.

Fluorometric enzymatic techniques were used to determine intermediates and substrates in perchloric acid extracts of skeletal muscle and in the incubation medium. Glucose (5), lactate (6), ATP (5), ADP (5), phosphocreatine (5), alanine (7), glutamate, and glutamine (2) were all assayed using coupled enzyme assays linked to the appearance or disappearance of NADH or NADPH. cAMP was determined in trichloracetic acid extracts of muscle by a sensitive double-antibody radioimmunoassay (8).

RESULTS

The effects of serotonin on alanine and glutamine formation and release in skeletal muscle were studied using the isolated, intact epitrochlaris preparations of rat skeletal muscle described previously (2). Increasing concentrations of serotonin progressively decreased alanine, glutamine, and glutamate release from skeletal muscle (Fig. 1A–C). At the highest concentration of serotonin studied (0.1 mM), alanine release (Fig. 1A) was reduced 36.8% ± 4.6% compared with control rates, glutamine release (Fig. 1B) was reduced 34.0% ± 3.9%, and glutamate release (Fig. 1C) was reduced 37.2% ± 5.1%. Serotonin addition concomitantly decreased tissue levels of alanine, glutamine, and glutamate compared with control incubations. However, tissue amino acid levels in control muscles after incubation were identical with tissue levels found in skeletal muscle freeze-clamped *in situ* (Table 1). At 10 μM, serotonin reduced muscle alanine levels from 1.49 ± 0.04 to 1.18 ± 0.10 μmoles/g; muscle glutamine levels, from 3.41 ± 0.17 to 2.58

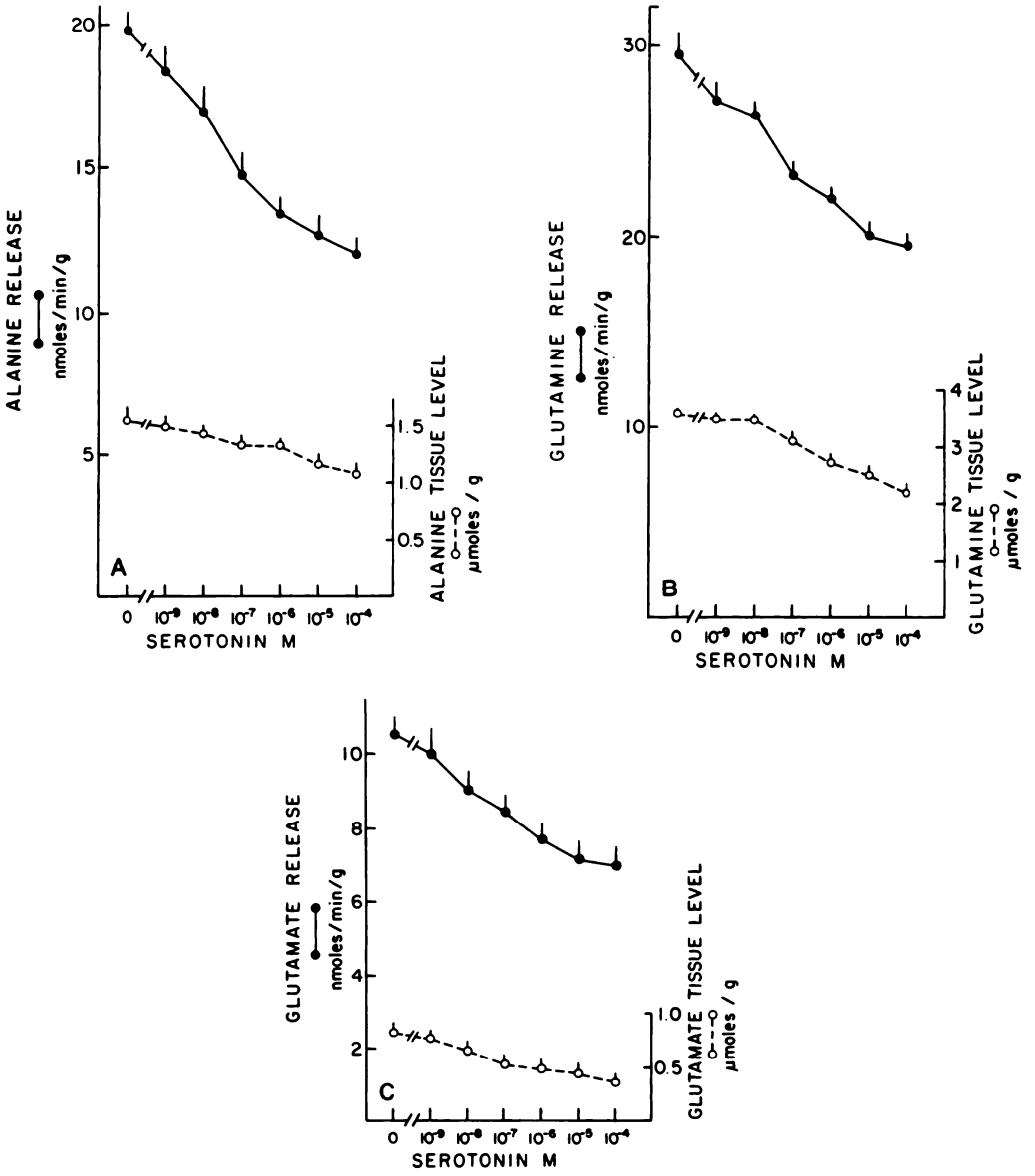

FIG. 1. *Effects of serotonin on release and tissue levels of skeletal muscle alanine (A), glutamine (B), and glutamate (C)*

Intact epitrochlaris preparations of rat skeletal muscle were incubated for 1 hr in Kreb-Henseleit buffer containing glucose (5 mM) as outlined in MATERIALS AND METHODS. Serotonin was added to the final concentrations indicated. Alanine, glutamine, and glutamate were determined in the medium after incubation. Muscles were frozen in liquid nitrogen immediately following incubation, and amino acid levels were determined enzymatically in the neutralized perchloric acid extracts of each of these preparations. Values for alanine, glutamine, and glutamate release are expressed as nanomoles per minute per gram of muscle, wet weight, and values for tissue levels are given as micromoles per gram.

± 0.21 μmoles/g; and muscle glutamate levels, from 0.81 ± 0.05 to 0.45 ± 0.05 μmol/g.

The serotonin-induced decreases in tissue level and rate of release of alanine in muscle could not be accounted for by a corresponding increase in the rate of muscle alanine utilization either by oxidative

TABLE 1

Effects of serotonin on alanine and glutamine balance in skeletal muscle before and after incubation

Intact epitrochlaris preparations of rat skeletal muscle were obtained and incubated for 1 hr at 37° under an atmosphere of 95% O_2–5% CO_2 in 0.5 ml of Krebs-Henseleit buffer (pH 7.4) containing HEPES (5 mM) and glucose (5 mM). Serotonin was added to a final concentration of 10 μM as indicated. Following incubation, muscles were rapidly removed, rinsed, blotted, and frozen in liquid nitrogen. Subsequently levels of alanine and glutamine were determined enzymatically in the neutralized perchloric acid extracts of these preparations. Alanine and glutamine released to the medium were also determined enzymatically. For comparative purposes, rat skeletal muscle was freeze-clamped *in situ* immediately following cervical dislocation of the animals, and alanine and glutamine were assayed in the perchloric acid extracts of these muscles. Values are means ± standard errors for at least six experiments.

Treatment	Alanine			Glutamine		
	Tissue level	Amount released	Total	Tissue level	Amount released	Total
	nmoles/mg muscle, wet wt			*nmoles/mg muscle, wet wt*		
Freeze-clamped *in situ*	1.52 ± 0.13			3.59 ± 0.26		
Control incubation	1.49 ± 0.04	1.18 ± 0.05	2.66 ± 0.06	3.41 ± 0.17	1.78 ± 0.11	5.22 ± 0.17
Serotonin	1.18 ± 0.10	0.78 ± 0.04	1.92 ± 0.08	2.53 ± 0.21	1.22 ± 0.15	3.75 ± 0.15

metabolism or by protein synthesis. The oxidation of [*U*-^{14}C]alanine to $^{14}CO_2$ and the rate of incorporation of alanine label (^{14}C) into trichloracetic acid-precipitable protein were determined using paired incubation studies (Table 2). In control studies, the rate of alanine oxidation amounted to less than 1% of the over-all alanine formation rate, and serotonin addition (10 μM) did not significantly increase this rate of alanine oxidation. In parallel experiments [*U*-^{14}C]alanine incorporation into trichloracetic acid-precipitable protein equaled only about 3% of the alanine release rate, and no increase in the rate of alanine incorporation into protein was produced by serotonin addition to the incubation medium. Similarly, serotonin did not alter rates of tritiated leucine incorporation into trichloracetic acid-precipitable protein in muscle preparations (Table 3). However, high concentrations of serotonin (10 μM) did produce a slight decrease in muscle levels of high-energy phosphorylated intermediates following incubation (Table 4). Phosphocreatine levels decreased 22.0%, from 12.2 ± 0.40 to 9.51 ± 0.63 μmoles/g, and ATP levels, 10.1%, from 4.05 ± 0.29 to 3.64 ± 0.21 μmoles/g, but the latter decrease was not statistically significant. Tissue levels of ADP and the calculated ratio of ATP to ADP were not significantly altered by serotonin. Those levels observed in preparations after incubation in control medium were not different from levels found in skeletal muscle freeze-clamped *in vivo* (2).

In order to characterize further the mechanism and specificity of the serotonin inhibition of alanine and glutamine formation and release in muscle, the effects of a number of serotonin antagonists were studied. Neither morphine sulfate nor low concentrations of tetrodotoxin reversed the serotonin-induced inhibition of alanine and glutamine release (Table 5). On the other hand, the simultaneous addition of either methysergide or cyproheptadine completely blocked the inhibition by serotonin of alanine and glutamine formation and release. None of these serotonin antagonists had significant independent actions on alanine and glutamine release in control incubations.

Previously we demonstrated that cAMP derivatives such as N^6-2′-*O*-dibutyryl cAMP decreased alanine and glutamine formation and release from skeletal muscle (1). For this reason, levels of muscle cAMP were determined after incubation in the presence and absence of serotonin. In a series of independent experiments (data not shown), maximal increments in steady-state concentrations of cAMP were found 5 min after incubation in serotonin-containing medium. Increased levels of

skeletal muscle cAMP were observed after 5 min of incubation with each concentration of serotonin studied (Fig. 2). Even at the lowest concentration used in these experiments (10 nM), a slight but statistically significant increase (14.5%) in cAMP levels was observed. At the highest concentration of serotonin (0.1 mM) cAMP was increased 150%.

If the mechanism of serotonin inhibition of alanine and glutamine release requires the intermediary participation of cAMP, inhibitors of cAMP phosphodiesterase should potentiate the inhibition of alanine and glutamine formation produced by submaximal concentrations of serotonin. Theophylline or 3-isobutyl- 1-methylxanthine addition slightly decreased alanine and glutamine formation (Table 6). However, the simultaneous addition of either of these cAMP phosphodiesterase inhibitors markedly augmented the inhibition of alanine and glutamine release produced by 1 μM serotonin alone. In a parallel series of experiments, steady-state muscle

TABLE 2

Effects of serotonin on alanine utilization by skeletal muscle

Epitrochlaris preparations of rat skeletal muscle were obtained and incubated as outlined in Table 1 and MATERIALS AND METHODS. In studies on [*U*-^{14}C]alanine oxidation, the incubation medium was modified to include glucose (5 mM), insulin (100 munits/ml), and alanine (0.5 mM). For studies of alanine incorporation into trichloracetic acid-precipitable protein, a mixture of 17 essential and nonessential amino acids (2.5 mM each) was added to the incubation medium as well as glucose (5 mM), insulin (100 munits/ml), and alanine (0.4 mM). [*U*-^{14}C]Alanine was added to each flask (1 μCi/ml). Evolution of $^{14}CO_2$ as well as the appearance of alanine label in trichloracetic acid-precipitable protein was determined after 1, 2, and 4 hr, using paired incubations. For final calculations of the rate of alanine flux both to CO_2 and to protein, mean specific activities for [*U*-^{14}C]alanine were determined using alanine specific activities prior to and after the conclusion of the incubations. Values are means ± standard errors for at least four experiments.

Incubation	[*U*-^{14}C]Alanine	
	Oxidation to $^{14}CO_2$	Reincorporation into acid-precipitable protein
	nmole/min/g muscle, wet wt	
Control	0.135 ± 0.017	0.623 ± 0.069
Serotonin, 10 μM	0.155 ± 0.023	0.677 ± 0.102

TABLE 3

Effect of serotonin in vitro on incorporation of tritiated leucine into skeletal muscle protein

Epitrochlaris preparations of rat skeletal muscle were obtained and incubated for 4 hr in modified Krebs-Henseleit buffer (pH 7.4) containing glucose (5 mM), a mixture of 17 essential and nonessential amino acids, [4,5-^{3}H]leucine (1.0 μCi), and insulin (100 munits/ml). After incubation, the muscles were rinsed, blotted, frozen in liquid nitrogen, and subsequently weighed. Tritium label incorporation into the washed trichloracetic acid precipitates of each muscle was determined following solubilization and liquid scintillation spectroscopy. Values are means ± standard errors for at least six experiments.

Incubation	[4,5-^{3}H]Leucine incorporation into acid-precipitable protein
	dpm/mg muscle
Control	9730 ± 1090
Serotonin, 10 μM	9782 ± 1196

TABLE 4

Effects of serotonin on skeletal muscle phosphocreatine, ATP, and ADP levels

Epitrochlaris preparations were obtained and incubated for 1 hr as outlined in Table 1 and MATERIALS AND METHODS. Serotonin was added to the incubation medium up to the final concentrations indicated. Following the conclusion of the incubation, muscles were rapidly removed from the medium, blotted, and immediately frozen in liquid nitrogen. Levels of phosphocreatine, ATP, and ADP were determined enzymatically in the neutralized perchloric acid extracts of these muscle preparations. Values are means ± standard errors for at least six experiments.

Addition	Phosphocreatine	ATP	ADP	ATP:ADP
	μmoles/g muscle, wet wt			
None	13.9 ± 1.01	4.17 ± 0.19	0.48 ± 0.02	8.10 ± 0.80
Serotonin, 0.1 μM	12.0 ± 1.10	4.05 ± 0.29	0.45 ± 0.04	7.72 ± 0.31
Serotonin, 10 μM	9.51 ± 0.63	3.64 ± 0.21	0.37 ± 0.04	8.46 ± 0.44

TABLE 5

Effects of serotonin antagonists on serotonin-induced inhibition of alanine and glutamine release from skeletal muscle

Epitrochlaris preparations of rat skeletal muscle were obtained and incubated as described in Table 1. Serotonin, morphine, tetrodotoxin, methysergide, or cyproheptadine was added as indicated. At the conclusion of the 1-hr incubation, muscles were removed from the medium and rapidly frozen in liquid nitrogen. Alanine and glutamine were determined enzymatically in the medium. Values are means ± standard errors for at least six experiments.

Addition	Alanine released		Glutamine released	
	−Serotonin	10 μM serotonin	−Serotonin	10 μM serotonin
	nmoles/min/g muscle, wet wt		*nmoles/min/g muscle, wet wt*	
None	20.0 ± 0.8	13.7 ± 0.6	29.5 ± 1.8	20.4 ± 0.9
Morphine sulfate, 1 μg/ml	20.2 ± 1.7	13.6 ± 0.9	20.8 ± 2.8	22.0 ± 1.1
Tetrodotoxin, 0.1 μg/ml	18.1 ± 1.3	13.4 ± 1.3	26.5 ± 1.2	18.6 ± 0.8
Tetrodotoxin, 1 μg/ml	18.0 ± 1.4	14.1 ± 1.4	27.9 ± 1.0	19.3 ± 1.9
Methysergide, 0.1 μM	22.1 ± 2.1	20.0 ± 2.9	30.8 ± 1.7	31.6 ± 2.6
Cyproheptadine, 0.1 μM	21.6 ± 2.1	23.2 ± 2.7	31.9 ± 3.1	33.5 ± 3.1

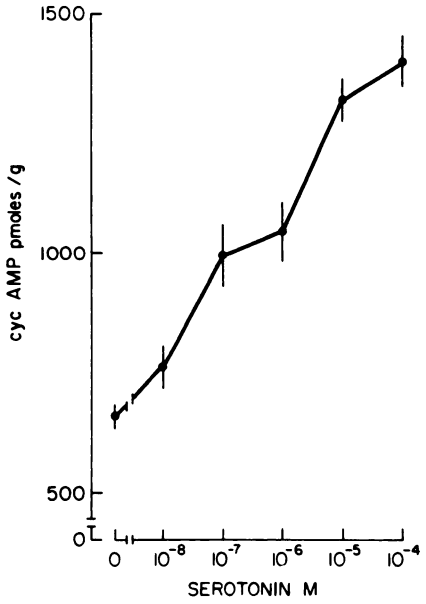

FIG. 2. *Effects of serotonin on steady-state levels of skeletal muscle cAMP*

Isolated intact epitrochlaris preparations of rat skeletal muscle were obtained and incubated as outlined in Table 1 and MATERIALS AND METHODS. Serotonin was added to the final concentrations indicated. Following a 10-min incubation, each muscle was rapidly removed from the medium and immediately frozen in liquid nitrogen. Using a double-antibody radioimmunoassay, cAMP was determined in trichloroacetic acid extracts of each muscle. Values are means ± standard errors for at least six experiments and are expressed as picomoles of cAMP per gram of muscle, wet weight.

cAMP levels were also determined (Table 7). Each xanthine alone increased muscle cAMP concentrations somewhat, as measured after 5 min of incubation. A moderate concentration of serotonin (1 μM) increased cAMP levels 72% relative to basal levels. The inclusion of theophylline (1 mM) together with serotonin (1 μM) amplified the increment in cAMP levels 2.5-fold, to a concentration which was 175% greater than basal. Isobutylmethylxanthine potentiated the serotonin-induced cAMP increment 3-fold, to a level 210% greater than basal.

In addition to the preceding observations on the augmentation of serotonin-induced increments in muscle cAMP levels, the effects of serotonin antagonists on muscle cAMP levels were investigated. As shown in Table 8, methysergide or cyproheptadine virtually abolished the increment in muscle cAMP produced by 10 μM serotonin. However, neither morphine nor low concentrations of tetrodotoxin modified the increase in muscle cAMP produced by serotonin.

The preceding studies on alanine and glutamine formation and release from skeletal muscle relied upon the utilization of endogenous amino acid substrates and their subsequent conversion to alanine and glutamine. Therefore the effect of serotonin on the conversion of exogenous amino acids to alanine and glutamine by

TABLE 6

Effects of cAMP phosphodiesterase inhibitors and serotonin on skeletal muscle alanine and glutamine release

Epitrochlaris preparations of rat skeletal muscle were obtained and incubated for 1 hr as described in Table 1 and MATERIALS AND METHODS. Theophylline, 3-isobutyl-1-methylxanthine, and serotonin were added to the incubation medium as indicated. Alanine and glutamine were determined enzymatically in the medium after incubation for 1 hr. Values are means ± standard errors for at least four experiments.

Inhibitor	Serotonin	Alanine release	Glutamine release
	μM	*nmoles/min/g muscle, wet wt*	
None	0	21.1 ± 1.5	32.4 ± 2.1
	1	13.8 ± 0.5	23.1 ± 1.7
Theophylline, 1 mM	0	18.2 ± 1.1	26.0 ± 1.3
	1	10.2 ± 0.9	16.0 ± 0.9
3-Isobutyl-1-methylxanthine, 0.2 mM	0	12.5 ± 2.0	21.7 ± 2.0
	1	6.9 ± 0.9	15.8 ± 2.7

TABLE 7

Effects of cAMP phosphodiesterase inhibitors on serotonin-induced increments in skeletal muscle cAMP levels

Epitrochlaris preparations were obtained and incubated for 5 min in Krebs-Henseleit buffer (pH 7.4) containing HEPES (5 mM) and glucose (5 mM). Serotonin, theophylline, and 3-isobutyl-1-methylxanthine were added as indicated. cAMP was determined by double-antibody radioimmunoassay of the trichloracetic acid extract of each muscle. Values are means ± standard errors for six experiments.

Inhibitor	Serotonin	cAMP
	μM	*pmoles/g muscle, wet wt*
None	0	611 ± 21
	1	1048 ± 68
Theophylline, 1 mM	0	831 ± 13
	1	1681 ± 67
3-Isobutyl-1-methylxanthine, 0.2 mM	0	1136 ± 79
	1	1894 ± 103

TABLE 8

Effects of selected serotonin antagonists on serotonin-induced increments in steady-state levels of cAMP in skeletal muscle

Epitrochlaris preparations were obtained and incubated for 5 min in Krebs-Henseleit buffer (pH 7.4) containing HEPES (5 mM) and glucose (5 mM). Serotonin, morphine, tetrodotoxin, methysergide, or cyproheptadine was added as indicated. After incubation, muscles were frozen in liquid nitrogen and subsequently homogenized in 12% trichloracetic acid. cAMP was determined by double-antibody radioimmunoassay of these extracts. Values are means ± standard errors for four experiments.

Antagonist	Serotonin	cAMP
	μM	*pmoles/g muscle, wet wt*
None	0	616 ± 19
	10	1459 ± 80
Morphine, 0.1 μg/ml	0	790 ± 76
	10	1548 ± 86
Tetrodotoxin, 0.1 μg/ml	0	755 ± 28
	10	1638 ± 105
Methysergide, 0.1 μM	0	622 ± 42
	10	610 ± 42
Cyproheptadine, 0.1 μM	0	692 ± 63
	10	738 ± 60

isolated muscle preparations was investigated. The addition to the medium of aspartate, cysteine, leucine, or valine (2 mM each) substantially increased the rate of alanine and glutamine release, but these increased rates were not altered by 10 μM serotonin in the incubation medium (Table 9).

These data suggest that serotonin may limit endogenous substrate amino acid availability, thereby limiting the rate of alanine and glutamine formation in muscle. One mechanism by which serotonin could decrease the intracellular supply of precursor amino acids is by decreasing the rate of degradation of endogenous skeletal muscle proteins. This hypothesis was assessed experimentally, using epitrochlaris preparations from animals previously injected with [*guanido*-^{14}C]arginine by a pulse-chase technique which results in partial labeling of muscle proteins (9). Although a small amount of recycling of label may occur with prolonged experiments *in vivo* (10), it is not a serious problem for such an assessment *in vitro*. Nevertheless, an amino acid was chosen for which the

carbon label has a relatively low recycling rate owing to the high activity of hepatic arginase (EC 3.5.3.1). Furthermore, arginine is not itself a substrate for muscle alanine or glutamine formation (3). The data obtained from incubated preparations were compared with contralateral preparations which were immediately frozen in liquid nitrogen after isolation (Table 10). Incubation in control medium produced a 15% decrease in the quantity of ^{14}C label in trichloracetic acid-precipitable protein, but this decrement was reduced to only 6% when serotonin (10 μM) was included in the medium. Correspondingly, incubation in control medium produced a 43% increase in the amount of nonprecipitable ^{14}C label (sum of the muscle post-trichloracetic acid supernatant and the incubation medium). In the presence of serotonin, however, this increment was only 21%. In terms of absolute numbers of disintegrations per minute per milligram of muscle, the decrease in ^{14}C labeling of muscle proteins following incubation was quantitatively accounted for by the increment in nonprecipitable label. Furthermore, the decreased amounts of label lost from protein during incubations in the presence of serotonin were equal to the small increases in nonprecipitable label in the incubation medium and in the acid-soluble fraction of muscle.

TABLE 9

Effects of serotonin on skeletal muscle alanine and glutamine conversion from added amino acids

Eptrochlaris preparations were obtained and incubated as detailed under MATERIALS AND METHODS. Aspartate, cysteine, leucine, or valine was added at a final concentration of 2.0 mM as indicated. Serotonin was also added as indicated. Following the conclusion of the 1-hr incubation, the muscles were rapidly removed from the medium and frozen in liquid nitrogen, and the medium subsequently was assayed enzymatically for alanine and glutamine content. Values are means ± standard errors for at least five experiments.

Amino acid added	Serotonin	Alanine release	Glutamine release
	μM	*nmoles/min/g muscle, wet wt*	
None	0	19.7 ± 0.8	29.5 ± 1.8
	10	12.9 ± 0.6	20.4 ± 0.8
Aspartate	0	56.5 ± 3.8	37.2 ± 1.9
	10	60.9 ± 2.6	36.4 ± 1.6
Cysteine	0	44.1 ± 2.9	43.9 ± 2.8
	10	43.8 ± 2.1	46.9 ± 2.2
Leucine	0	31.1 ± 2.6	39.9 ± 2.2
	10	29.1 ± 1.4	38.3 ± 1.4
Valine	0	34.8 ± 2.9	38.9 ± 1.1
	10	35.2 ± 2.7	40.9 ± 2.5

DISCUSSION

The results of the present study demonstrate clearly that serotonin inhibits alanine and glutamine formation and release from skeletal muscle. No direct effects of

TABLE 10

Effect of incubation with serotonin on distribution of [^{14}C]arginine in vitro from skeletal muscle proteins previously labeled in vivo

Epitrochlaris preparations were obtained from animals previously injected with [*guanido*-^{14}C]-arginine as outlined under MATERIALS AND METHODS. Muscles from one extremity were freeze-clamped immediately following their isolation, and muscles from the contralateral extremity were incubated for 2 hr in Krebs-Henseleit buffer (pH 7.4) with serotonin (10 μM) added as indicated. Following incubation, muscle preparations were rapidly frozen in liquid nitrogen. Trichloracetic acid-precipitable protein was obtained from each muscle preparation, washed twice, and subsequently isolated and counted using liquid scintillation spectrometry. The muscle supernatants following trichloracetic acid treatment were sampled directly for liquid scintillation counting, as was the incubation medium where appropriate. Values are means ± standard errors for at least eight experiments.

Treatment	Muscle		Released to medium	Total	Increment in nonprecipitable label
	Acid-insoluble	Acid-soluble			
	dpm/mg muscle, wet wt				
Frozen, unincubated	168.2 ± 3.1	57.2 ± 1.6		226.3 ± 8.9	
Control incubation	142.5 ± 3.7	28.0 ± 3.0	54.1 ± 3.2	225.1 ± 7.8	24.9
Serotonin	158.4 ± 2.4	29.6 ± 2.0	39.8 ± 3.7	228.1 ± 8.2	12.2

serotonin on skeletal muscle metabolism have previously been reported. The reduction in alanine and glutamine release produced by serotonin appears to result from a decreased rate of alanine and glutamine formation and not from inhibition of amino acid transport. Four different observations support this conclusion. First, tissue alanine and glutamine levels were decreased rather than increased by serotonin. Since alanine and glutamine are synthesized within the muscle cell, primary inhibition of their transport should have increased intracellular amino acid levels to the same extent as extracellular levels decreased. Such was not observed (Table 1). Second, alanine re-entry and back-transport are processes essential for studies of [*U*-^{14}C]alanine oxidation to $^{14}CO_2$ or incorporation into proteins (Table 2). Since transport can be rate-limiting for either metabolic pathway, a significant effect of serotonin on alanine re-entry can be excluded, based on the absence of serotonin-induced inhibition of [*U*-^{14}C]alanine oxidation or incorporation into protein. Third, [4,5-^{3}H]leucine entry and incorporation into muscle protein were also unaltered by serotonin (Table 3). Fourth, the transport and conversion of added, extracellular amino acids (Table 9) to alanine and glutamine were also unimpaired by serotonin. Again, amino acid transport can be a rate-limiting process for alanine and glutamine synthesis (4), and the lack of effect of serotonin on these complex processes provides good evidence that the mechanism of serotonin action is not limited to a reduction of amino acid transport. Instead, it seems reasonable to conclude that serotonin reduces alanine and glutamine formation in skeletal muscle. These effects of serotonin appear to be relatively specific and require the participation of a D-class serotonin receptor, since they were blocked by cyproheptadine and methysergide but not by morphine sulfate or tetrodotoxin (12). In addition to the participation of a D receptor, the metabolic action of serotonin appears to be mediated by intracellular cAMP. Four lines of experimental evidence support this conclusion. (a) Concentrations of serotonin inhibiting amino acid release also produced an increased steady-state level of cAMP in skeletal muscle (Fig. 2). (b) Incubation of muscles with serotonin together with cAMP phosphodiesterase inhibitors substantially amplified the serotonin-induced increments in muscle cAMP levels and the serotonin-induced inhibition of amino acid release (Tables 6 and 7). (c) Both cyproheptadine and methysergide blocked the metabolic effects of serotonin on amino acid release and the serotonin-induced increments in cAMP levels, whereas morphine and tetrodotoxin blocked neither effect of serotonin (Tables 5 and 8). (d) Dibutyryl cAMP addition alone reproduced the serotonin-induced inhibition of alanine and glutamine formation and release from skeletal muscle (1). In addition to these qualitative interrelationships between serotonin-induced cAMP accumulation and inhibition of amino acid release, a proportional relationship between the extent of the increase in cAMP and the decrease in amino acid release seems generally evident. For example, the progressive concentration-dependent decrease in alanine and glutamine release (Fig. 1A and B) was mirrored almost exactly by a progressive, concentration-dependent increase in cAMP levels (Fig. 2).

Serotonin does not appear to be as potent an agonist as does epinephrine in the epitrochlaris preparations. At equimolar concentrations of each (10 μM), serotonin increased steady-state muscle cAMP levels from 610 ± 27 to 1459 ± 80 pmoles/g, but epinephrine increased cAMP levels to 2237 ± 214 pmoles/g. It is also interesting to note that the smaller increments in cAMP levels produced by serotonin were associated with less inhibition of alanine and glutamine formation. For example, serotonin (10 μM) decreased alanine and glutamine formation and release 35.6% and 34.8%, respectively, whereas epinephrine (10 μM) decreased alanine and glutamine formation and release 48.0% and 46.2%, respectively (1). These observations provide additional support for the concept that the mechanism of serotonin action on amino acid formation and release in skeletal muscle requires the intermediary par-

ticipation of cAMP. Prior studies on cyclic nucleotide mediation of serotonin action have yielded conflicting results. In human monocytes (13) and rat uterine smooth muscle (14), serotonin has been found to induce the accumulation of cGMP. On the other hand, in rat myocardium (15), rat adrenal cells (16), and molluscan and fluke muscle (17–20), serotonin clearly increased levels of cAMP but not of cGMP. In the present study with rat skeletal muscle, serotonin clearly increased cAMP levels, and the metabolic effects of serotonin appear to be related to the observed increments in cAMP.

There are four potential mechanisms by which the serotonin-induced increments in muscle cAMP could result in a diminution of alanine and glutamine formation and release from skeletal muscle. These include (a) an increased rate of alanine and glutamine oxidation in skeletal muscle, (b) an increased rate of alanine and glutamine reutilization for protein synthesis in muscle, (c) direct inhibition of one or more steps involved in the conversion of precursor amino acids to alanine and glutamine, or (d) inhibition of the rate of degradation of endogenous muscle proteins, thereby decreasing the availability of substrate amino acids for subsequent conversion to alanine and glutamine. That serotonin does not increase the rate of muscle reutilization of alanine by protein synthesis or by oxidative metabolism is shown clearly by the data of Table 2. In addition, serotonin did not alter rates of protein synthesis in muscle as judged by the incorporation of tritiated leucine into trichloracetic acid-precipitable protein (Table 3). Of the remaining mechanisms which could account for the inhibition of muscle alanine and glutamine formation, it seems most probable that the mechanism of serotonin action involves a reduction in the rate of muscle proteolysis. This conclusion derives in part from the finding that the rate of conversion of exogenous amino acids such as aspartate, cysteine, leucine, and valine to alanine and glutamine was insensitive to inhibition by serotonin (Table 9). Further support is provided by the data of Table 10. In these experiments to assess directly the rate of muscle proteolysis *in vitro*, its seems evident that serotonin reduced the solubilization of amino acid label in muscle protein. These data provide additional evidence that the mechanism by which the serotonin-induced increases in cAMP levels inhibit alanine and glutamine formation involves a primary reduction in the rate of skeletal muscle proteolysis. Such a reduction thereby limits the intracellular availability of precursor amino acids for subsequent conversion to alanine and glutamine. These studies, however, do not elucidate further the precise enzymatic steps or cellular organelles participating in pathways of skeletal muscle protein degradation which might be modified by serotonin- or epinephrine-induced increments in levels of cAMP.

Interpretations concerning the physiological relevance of these observations that serotonin inhibits alanine and glutamine formation in skeletal muscle depend in part upon the demonstration that effective concentrations of serotonin *in vitro* are consistent with known serotonin concentrations *in vivo*. Circulating levels of serotonin in human serum, as determined by a variety of fluorometric and bioassay methods, range between 0.11 and 0.48 μM (21–23). This concentration *in vivo* is entirely consistent with the range of serotonin concentrations associated with inhibition of muscle alanine and glutamine formation *in vitro*. Augmentation of circulating concentrations of serotonin is possible by activation and extrusion of platelet serotonin storage granules, since in packed platelets serotonin exists at a maximal concentration of 130 μM (24, 25). In addition, modulation of local serotonin concentrations may result from variations in the rate of discharge of serotonergic neurons of the autonomic nervous system (26).

These results clearly demonstrate that serotonin has a direct effect on skeletal muscle metabolism, causing a substantial decrease in the rate of alanine and glutamine formation that apparently is secondary to retarding effects on the rate of muscle proteolysis. The mechanism of serotonin action requires specific D receptors

in muscle and the intermediary participation of intracellular levels of cAMP.

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