ratio of bound/free CPK and consequently with optimal energy utilization in cardiac tissue.

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Decreases in the release of acetylcholine *in vitro* with low oxygen

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A decrease in the O_2 content of the inspired air (hypoxia) depresses higher integrative function in man and animals [1–3] but does not reduce brain energy reserves [4–8]. Thus, a decrease in the metabolism of neurotransmitters has been postulated as a possible molecular basis of hypoxic brain dysfunction [9]. Hypoxia impairs the *in vivo* synthesis of acetycholine (ACh)* [8, 10, 11], serotonin [12], the catecholamines [13] and the amino acids [11, 14, 15], while their concentrations remain unaffected. The decrease in synthesis during hypoxia without a corresponding reduction in levels implies that hypoxic insults may alter release mechanisms and that the non-released neurotransmitter may impair further synthesis. Indirect evidence suggests that low O_2 may impair dopamine release [16]. We tested this hypothesis directly with the cholinergic system, and found that a decrease in O_2 inhibits the Ca^{2+}-dependent release of ACh from brain slices and synaptosomes. Since the effects of hypoxia on release were dependent on the presence of Ca^{2+}, we tried to ameliorate the inhibitory effects of hypoxia with a pharmacological agent that interacts with Ca^{2+} homeostasis. 4-Aminopyridine increases neurotransmitter release in a Ca^{2+}-dependent manner [17, 18]. It increases the influx of Ca^{2+} into nerve terminals [19–21] although this increase may be secondary to alterations in K^+ channels [22, 23].

All reagents and procedures were as previously described [24]. Brain slices were prepared from male CD-1 mice (18–25 g). Slices (2–3 mg protein) were preincubated under 100% O_2 with 5 mM [U-^{14}C]glucose (1 μCi/μmole). After

* Abbreviations: ACh, acetylcholine; CDKS, Ca^{2+}-dependent-K^+-stimulated; EGTA, ethylene glycol-bis[β-aminoethylether]-*N*,*N*,*N*′,*N*′-tetraacetic acid; and 4-AP, 4-aminopyridine.

the preincubated slices were rinsed with the appropriate release buffer [i.e. Ca^{2+} (Ca^{2+}-dependent release) or 1 mM EGTA, pH 7.4 (non-Ca^{2+}-dependent release)], they were flushed for 10 min (10 l/min) with 100%, 2.5% or 0% O_2. KCl (26 μmoles to give a final concentration of 31 mM) and/or 0.1 mM 4-aminopyridine (pH 7.4) were added to the appropriate tubes. The 10-min release incubation and the extraction procedure were exactly as previously described [24].

Synaptosomes were prepared from mouse brain by the method of Booth and Clark [25] with two modifications. Tris–HCl (5 mM, pH 7.4) was included in the Ficoll solutions. The final concentration of the lower Ficoll layer was 10.5% rather than 10% which helped to produce more distinct banding. The purified synaptosomal band was removed and diluted 1:4 (v/v) in the same ice-cold buffer that was used with the brain slices. The synaptosomes were centrifuged (4000 *g* for 10 min at 4°) and then resuspended and rinsed twice with the same buffer. The glucose concentration in the media was reduced to 1.5 mM (3.3 μCi/μmole) for preincubation to allow adequate [U-^{14}C]glucose incorporation into ACh [26]. The 10-min release protocol followed that described for slices.

Multiple statistical comparisons were done with Student's *t*-test. So that the possibility of type 2 errors was minimized, we reduced the level of significance to $P < 0.001$ [27].

Low O_2 decreased the Ca^{2+}-dependent release of ACh. With 100% O_2, nearly 80% of the [^{14}C]ACh that was released from preincubated slices was Ca^{2+}-dependent and stimulated by K^+ depolarization (Table 1). If the O_2 content was lowered to 2.5% or 0% O_2, the Ca^{2+}-dependent-K^+-stimulated (CDKS) release of [^{14}C]ACh declined 55 and 60% respectively. Neither the high-K^+-non-Ca^{2+}-dependent release nor the resting release with or without Ca^{2+} was dependent on the concentration of O_2. The (high K^+ minus the low K^+) Ca^{2+}-dependent release decreased 56 and 66% with 2.5% and 0% O_2 respectively. About 50% of the [^{14}C]ACh released from preincubated synaptosomes was CDKS with 100% O_2 (Table 2). The CDKS release of [^{14}C]ACh decreased 23 and 30% with 2.5% and 0% O_2 respectively. The high-K^+-Ca^{2+}-dependent release decreased while the low K^+-Ca^{2+}-dependent release increased nonsignificantly. The (high K^+ minus the low K^+) Ca^{2+}-dependent release decreased 33 and 58% with 2.5% and 0% O_2.

4-Aminopyridine partially reversed the inhibitory effects of low O_2 on the Ca^{2+}-dependent release of ACh. When hypoxic slices were incubated with 4-aminopyridine, the decrease in CDKS release with 2.5% O_2 was reduced from 55 to 24% and the 60% inhibition under 0% O_2 diminished to 27% (Table 1). Synaptosomes responded similarly to pharmacological manipulation since the 23% inhibition with 2.5% O_2 was completely reversed and that with 0% O_2 improved 21% (Table 2). 4-Aminopyridine increased the Ca^{2+}-dependent release with low K^+ to that observed with high K^+ under identical O_2 tensions. 4-Aminopyridine was ineffective at all O_2 concentrations if Ca^{2+} was omitted from the release incubation media.

The decreased CDKS release may account for the depressed synthesis of [^{14}C]ACh observed with hypoxia. When [U-^{14}C]glucose was added just to the release incubation, only 15% of the total CDKS releasable [^{14}C]ACh (dpm/mg protein) was synthesized during that 10 min period. The total synthesis of [^{14}C]ACh during a 10 min incubation with 0% O_2 decreased 54% while the inhibition of release was 60%.

The decreased Ca^{2+}-dependent release of ACh with hypoxia and the stimulation of release with 4-aminopyridine only in the presence of Ca^{2+} suggest that low O_2 may alter brain function by interfering with the role of Ca^{2+} in release. Low O_2 may alter cellular Ca^{2+} distribution by several mechanisms. Hypoxia reduces mitochondrial pyridine nucleotides [8] which may subsequently block the efflux of Ca^{2+} from the mitochondria [28]. Ca^{2+} uptake into stimulated smooth muscle is reduced by low O_2 [29]. Hypoxia-like anesthetics may impair the depolarization-triggered Ca^{2+} entry into presynaptic terminals [30].

A cholinergic deficit may contribute to the biochemical [6], physiological [31], and psychological [32] alterations in brain function with hypoxia. *In vivo*, ACh synthesis declines with hypoglycemia [8], thiamine deficiency [33, 34] and histotoxic, anemic or hypoxic hypoxia [8, 10, 11]. *In vitro*, metabolic inhibitors and decreased O_2 tensions reduce oxidative metabolism and ACh synthesis proportionately even though less than 1% of the oxidized substrate is converted to ACh [10, 11, 22, 35]. Low O_2 blocks cholinergic ganglionic transmission while energy metabolism [36] and axonal conduction [37] remain normal. Pharmacological inhibition of the cholinergic system with scopolamine mimics hypoxic behaviour [38, 39] while cholinomimetics (e.g. physostigmine) delay the onset of the symptoms of hypoxic hypoxia [40] or chemical hypoxia [8]. The inhibition of ACh release by low O_2 may underlie the decrease in cholinergic function which occurs in the brain during hypoxia. Thus, manipulations which stimulate ACh release should prove efficacious in the treatment of the physiological symptoms of hypoxia.

In conclusion, low O_2 decreases the Ca^{2+}-dependent release of ACh during depolarization. This inhibition occurs by a mechanism which can be partially reversed by 4-aminopyridine only in the presence of Ca^{2+}. Whether or not low O_2 similarly decreases the release of other neurotransmitters and how Ca^{2+}, ACh release and the decline in mental function interact during hypoxia require further investigation.

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Table 1. ACh release from mouse brain slices with various O_2 tensions in the presence or absence of 4-aminopyridine*

Buffer conditions	[^{14}C]ACh [dpm · (mg protein)$^{-1}$ · 10 min^{-1}]					
	Gas composition 100% O_2		2.5% O_2		0% O_2	
	Minus 4-AP	Plus 4-AP	Minus 4-AP	Plus 4-AP	Minus 4-AP	Plus 4-AP
High K^+ plus Ca^{2+}	351.4 ± 7.8	380.7 ± 6.3	214.5 ± 8.1†‡	300.9 ± 27.3	180.6 ± 3.3‡§	308.0 ± 1.0
High K^+ minus Ca^{2+}	75.1 ± 4.6	84.5 ± 2.1	102.7 ± 6.6†	91.4 ± 6.5	70.3 ± 6.1	107.9 ± 9.6†
High K^+-Ca^{2+}-dependent (CDKS) release	276.8 ± 6.6 (100%)	296.0 ± 4.3 (107%)	123.7 ± 5.9†‡ (45%)	209.5 ± 21.9† (76%)	110.4 ± 4.2‡§ (40%)	200.1 ± 9.1† (73%)
Low K^+ plus Ca^{2+}	85.3 ± 3.9‡	370.4 ± 3.7†	78.6 ± 2.6‡	292.4 ± 4.3†	75.7 ± 4.6‡	300.7 ± 6.2†
Low K^+ minus Ca^{2+}	60.4 ± 3.6	78.4 ± 5.1	55.3 ± 5.6	85.9 ± 6.2†	52.7 ± 6.8	83.1 ± 5.2†
Low K^+-Ca^{2+}-dependent release	20.9 ± 2.1‡	290.7 ± 3.5†	22.1 ± 2.1‡	202.4 ± 3.5†	22.9 ± 3.1‡	217.8 ± 6.1†
High K^+ minus low K^+ Ca^{2+}-dependent release	258.5 ± 4.5 (100%)		114.9 ± 4.4† (44%)		87.7 ± 3.1§ (34%)	
(*N*)	(36)	(6)	(18)	(6)	(15)	(6)

* Mouse brain slices were preincubated for 1 hr with [U-^{14}C]glucose in low K^+ phosphate buffer with 100% O_2. The prelabelled [^{14}C]ACh was released during a 10-min incubation with high (31 mM) or low (5 mM) KCl, with Ca^{2+} or without Ca^{2+} (1 mM EGTA) and in the presence or absence of 0.1 mM 4-AP (4-aminopyridine). The O_2 tension (100%, 2.5% or 0%) was varied in each condition. The percentages in parentheses are the percentages of the Ca^{2+}-dependent release with 100% O_2 minus 4-AP. The *N* at the bottom of each column represents the number of individual samples that were measured in each separate condition (e.g. high K^+ minus Ca^{2+}). Slices were not used to measure more than one condition. Each experiment examined the indicated level of O_2 with or without 4-aminopyridine in triplicate per condition and was repeated at least twice on different days. Values are the means ± S.E.M. of the number of samples in parentheses. Significant differences were determined by Student's *t*-test ($P < 0.001$). The degrees of freedom for the *t*-test were obtained by adding the $(n-1)$ from each of the two conditions that were compared.

† Value significantly different from 100% O_2 minus 4-AP.

‡ Value significantly different from its corresponding gas-treated sample plus 4-AP.

§ Value significantly different from 100% and 2.5% O_2 minus 4-AP.

Table 2. ACh release from mouse brain synaptosomes with various O_2 tensions in the presence or absence of 4-aminopyridine*

Buffer conditions	[^{14}C]ACh [dpm · (mg protein)$^{-1}$ · 10 min^{-1}]					
	Gas composition: 100% O_2		2.5% O_2		0% O_2	
	Minus 4-AP	Plus 4-AP	Minus 4-AP	Plus 4-AP	Minus 4-AP	Plus 4-AP
High K^+ plus Ca^{2+}	226.0 ± 3.2	229.6 ± 8.3	176.3 ± 2.7†‡	213.5 ± 5.2	176.7 ± 2.4†	189.6 ± 7.6†
High K^+ minus Ca^{2+}	103.2 ± 2.7	95.4 ± 7.2	83.8 ± 4.8†	90.8 ± 2.1	90.7 ± 2.0	78.5 ± 4.9
High K^+-Ca^{2+}-dependent (CDKS) release	121.1 ± 2.3 (100%)	134.3 ± 4.6 (111%)	93.6 ± 6.9†‡ (77%)	121.4 ± 1.7 (100%)	85.3 ± 2.6†‡ (70%)	110.8 ± 3.0 (91%)
Low K^+ plus Ca^{2+}	66.7 ± 4.3‡	220.3 ± 10.7†	70.1 ± 8.7‡	207.4 ± 7.4†	106.7 ± 6.9‡	188.2 ± 9.0†
Low K^+ minus Ca^{2+}	59.2 ± 3.4‡	94.9 ± 7.2†	53.4 ± 8.1‡	92.7 ± 1.6†	75.2 ± 3.0†	84.4 ± 6.3†
Low K^+-Ca^{2+}-dependent release	6.7 ± 0.9‡	125.3 ± 17.6†	16.7 ± 2.5†‡	114.7 ± 6.9†	37.8 ± 2.6‡§	103.8 ± 3.1†
High K^+ minus low K^+ Ca^{2+}-dependent release	114.2 ± 1.8 (100%)		77.0 + 6.7† (67%)		47.4 ± 4.4§ (42%)	
(*N*)	(21)	(6)	(9)	(6)	(15)	(6)

* Mouse brain synaptosomes were preincubated for 1 hr with [U-^{14}C]glucose in low K^+ phosphate buffer with 100% O_2. The prelabelled [^{14}C]ACh was released during a 10-min incubation with high (31 mM) or low (5 mM) KCl, with Ca^{2+} or without Ca^{2+} (1 mM EGTA) and in the presence or absence of 0.1 mM 4-AP (4-aminopyridine). The O_2 tension (100%, 2.5% or 0%) was varied in each condition. The percentages in parentheses are the percentages of the Ca^{2+}-dependent release with 100% O_2 minus 4-AP. The *N* at the bottom of each column represents the number of individual samples that were measured in each separate condition (e.g. high K^+ minus Ca^{2+}). Synaptosomes were not used to measure more than one condition. Each experiment examined the indicated level of O_2 with or without 4-aminopyridine in triplicate per condition and was repeated at least twice on different days. Values are the means ± S.E.M. of the number of samples in parentheses. Significant differences were determined by Student's *t*-test ($P < 0.001$). The degrees of freedom for the *t*-test were obtained by adding the $(n - 1)$ from each of the two conditions that were compared.

† Value significantly different from 100% O_2 minus 4-AP.

‡ Value significantly different from its corresponding gas-treated sample plus 4-AP.

§ Value significantly different from 100% and 2.5% O_2 minus 4-AP.

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In situ formation of the acetaminophen metabolite covalently bound in kidney and lung Supportive evidence provided by total hepatectomy

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Acetaminophen may produce extrahepatic, as well as hepatic, lesions in rodents [1–3], and in humans [4]. After administration of acetaminophen, a metabolite covalently bound to macromolecules is found mainly in the liver but also in extrahepatic organs [1–3]. It is not yet clear, however, whether the metabolite bound in extrahepatic tissues is directly formed *in situ* [1, 2] or is formed in the liver and exported elsewhere [3]. Although the liver is probably the main site of formation of the reactive metabolite in the body, the metabolite may be too unstable to leave the liver and reach extrahepatic organs. McMurtry, Snodgrass and Mitchell [1] reported that pretreatment of male Fischer rats with 3-methylcholanthrene increased the *in vitro* covalent binding of the reactive metabolite of acetaminophen to hepatic microsomes but not to renal microsomes. This pretreatment increased the *in vivo* covalent binding to hepatic proteins but not to renal proteins [1]. It was suggested that the reactive metabolite covalently bound to proteins in the kidney did not come from the liver but was instead directly formed in the kidney [1]. In this communication, we report the effect of total hepatectomy on the *in vivo* covalent binding of the reactive metabolite of acetaminophen in kidney and lung. Results obtained by this different approach confirm the view that bound metabolites are mainly formed *in situ*.

Unlabeled acetaminophen was purchased from Sigma Chemical Co. (St Louis, MO). [^{3}H]Acetaminophen (generally labeled, sp.act. 5.6 Ci/mmole) was purchased from New England Nuclear (Boston, MA). Its radiochemical purity, checked by thin-layer chromatography, was found to be higher than 99%. Male Sprague–Dawley rats were purchased from Charles River, Saint-Aubin-lès-Elbeuf, France. Rats were fed a standard diet (Autoclavé 113, UAR, Villemoisson-sur-Orge, France) given *ad lib.* Animals were operated upon under ethyl ether anaesthesia. Total hepatectomy was performed in 3 stages as previously reported [5]; a catheter was inserted in the inferior vena cava and an infusion of glucose was started; another catheter was placed in a femoral artery. Control rats were subjected to a laparotomy and catheters were placed in the inferior vena cava and in a femoral artery. Animals weighed 300–360 g at the time of hepatectomy or laparotomy. Hepatectomized or laparotomized rats were held in restraining cages that were placed in heating chambers where the ambient temperature was automatically adjusted to maintain a rectal temperature of 37.5°. Hepatectomized rats remained in good hemodynamic and general condition during the whole period of the metabolic study.

The administration of acetaminophen was started 10 min after the completion of surgery. Acetaminophen was dis-