Radioimmunoassay of carbonic anhydrase VI in saliva and sheep tissues

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A specific and sensitive radioimmunoassay has been developed for the measurement of the secreted carbonic anhydrase isoenzyme (CA VI) in sheep saliva and tissues. The assay can detect as little as 75 pg of CA VI, and the antibody used does not cross-react with CA II or CA III. The intra-assay variation, measured using a saliva sample, was 3.0 %, whereas the inter-assay variation was 10.5 %. The concentration of CA VI in parotid saliva from normal, resting sheep was $5.6 \pm 3.0 \mu\text{g} \cdot \text{ml}^{-1}$ ($n = 42$) or $79.4 \pm 35.7 \mu\text{g} \cdot \text{mg}$ of total protein $^{-1}$. With feeding, the CA VI concentrations increased an average of 6-fold. The secretion rate of CA VI from the vascularly isolated neurotomized parotid gland of the anaesthetized sheep was $0.62 \pm 0.40 \mu\text{g} \cdot \text{min}^{-1}$, compared with a rate of $11.7 \pm 7.8 \mu\text{g} \cdot \text{min}^{-1}$ from the parotid gland of normal conscious sheep. Stimulation of the parotid-gland preparation by the muscarinic agent bethanechol increased the secretion rate to $438 \pm 172 \mu\text{g} \cdot \text{min}^{-1}$ ($n = 8$), and electrical stimulation of the secretomotor Moussu nerve increased CA VI secretion rate to $634 \pm 330 \mu\text{g} \cdot \text{min}^{-1}$ ($n = 4$). Submandibular saliva from anaesthetized sheep contained $6.9 \pm 2.1 \mu\text{g}$ of CA VI $\cdot \text{ml}^{-1}$ ($n = 3$). The only tissues found to contain measurable amounts of CA VI were the parotid ($6.4 \mu\text{g} \cdot \text{mg}$ of protein $^{-1}$) and submandibular ($1.8 \mu\text{g} \cdot \text{mg}$ of protein $^{-1}$) salivary glands. The sublingual salivary gland, kidney, lung, adrenal, brain, skeletal muscle, liver, heart, pancreas, small intestine and cerebrospinal fluid did not have a measurable CA VI content.

INTRODUCTION

Of the isoenzymes of carbonic anhydrase (CA) described in mammals, four isoenzymes are cytoplasmic (I, II, III and VII) [1], one occurs in the mitochondrial matrix (V) [2], one is membrane-bound (IV) [3] and only CA VI is secreted. CA VI is synthesized in the acinar cells of the parotid and submandibular salivary glands and is secreted into saliva [4]. The enzyme has been isolated from the sheep parotid gland and characterized [5] and its complete amino acid sequence was determined [6]. The rat [7] and human [8] enzymes also have been purified to homogeneity and characterized. This isoenzyme differs significantly from the cytoplasmic forms of the enzyme in its properties. The secreted enzyme is a glycoprotein with an apparent subunit molecular mass of about 45 kDa [4,7,8]. The sheep enzyme contains an extra 47 or 48 amino acids compared with the cytoplasmic isoenzymes, with most of these residues located in a C-terminal extension. CA VI has a sequence identity of only about 30 % with the cytoplasmic isoenzymes. In common with many extracellular proteins, CA VI is stabilized by a disulphide bond [6].

It was not known what the normal concentrations of this enzyme were in saliva, nor whether it was secreted constitutively or if its secretion was regulated. To address these questions, we have developed a specific and sensitive radioimmunoassay to measure CA VI concentrations in saliva and tissues of the sheep and report effects of nerve and pharmacological stimulation on CA VI secretion and tissue content.

EXPERIMENTAL

Purification of CA VI

CA VI was purified to homogeneity from sheep saliva as described previously [4,6]. Briefly, this involves passing filtered saliva through a sulphonamide–Sephacrose affinity resin and further purifying the eluted CA VI on wheat-germ-lectin–

Sephacrose and Sepharose 6B. The concentration of CA VI used for standards in the radioimmunoassay and the total protein in saliva and tissue samples were determined by a modified Lowry procedure [9], with BSA (ICN Pharmaceuticals) as standard.

Antibody

Antibody to sheep CA VI was raised in rabbits as previously described [5]. Purified antigen (0.5 mg/rabbit) emulsified with Freund's adjuvant was injected subcutaneously at monthly intervals, and bleeds were taken 2 weeks after the injections. The second antibody for the radioimmunoassay, anti-rabbit IgG, was raised in goats.

Labelling of antigen

Pure CA VI (20 μg) was iodinated using the [^{125}I]-Bolton–Hunter reagent [10] from Amersham International (2000 Ci $\cdot \text{mmol}^{-1}$). The labelled protein was purified by gel filtration on Sepharose 6B (Pharmacia) on a 1 cm $\times$ 30 cm column in 50 mM-phosphate buffer, pH 7.4, containing 0.25 % gelatin and 0.04 % $\text{Na}_2\text{S}_2\text{O}_3$. The tracer was stored at 4 °C.

Radioimmunoassay procedure

Standards and samples (maximum volume 100 μl), antibody (working dilution 1 in 5000; 100 μl) and [^{125}I]-CA VI (about 5000 c.p.m. in 300 μl of buffer) were incubated overnight at 4 °C. The buffer used was 50 mM-phosphate, pH 7.4, containing 0.1 % BSA (C.S.L., Parkville, Australia). To separate bound and free tracer, 100 μl of goat anti-rabbit IgG (1 in 20 dilution), 100 μl of normal rabbit serum (1 in 200 dilution) and 100 μl of 20 % poly(ethylene glycol) 6000 (BDH Chemicals) were added, the tubes incubated for a further 2 h at 4 °C, then centrifuged (3000 g, 30 min). Standards and samples were incubated in triplicate. Bound tracer was counted for radioactivity in a Packard Cobra 5010 γ -counter and the results calculated from a logit–log plot using computer analysis.

Abbreviations used: CA VI, CA III and CA II, carbonic anhydrase isoenzymes VI, III and II; VIP, vasoactive intestinal peptide.

Sample collection

Parotid saliva samples were collected from mature conscious Merino and cross-bred Merino sheep by cannulation of the parotid duct as described previously [11]. Preparation of the vascularly isolated neurotomized parotid gland in anaesthetized sheep was carried out as described elsewhere [12,13]. The gland was stimulated by infusion of bethanechol (Merck, Sharp and Dohme) in Macrodex (Pharmacia) into the carotid artery, giving a final concentration of about 5×10^{-6} M, or by electrical stimulation of Moussu's nerve [12]. The effect of vasoactive intestinal peptide (VIP; Peninsular Laboratories) on CA VI secretion was tested by infusing the peptide at a rate sufficient to give a final concentration of $(2-5) \times 10^{-9}$ M in the carotid artery, either by itself or in conjunction with the bethanechol infusion. Tissue samples were taken from the experimental parotid gland before and after stimulation and were homogenized in 0.1 M-phosphate buffer, pH 7.4, containing 5 mM-EDTA, 5 mM-benzamidine and 1 μ g of aprotinin $\cdot$ ml $^{-1}$ (Boehringer-Mannheim). After centrifugation, the supernatants were assayed for CA VI content and the total protein determined by the modified Lowry assay [9]. Similar tissue samples were processed to demonstrate histochemically CA activity by the technique of Coquin-Carnot and Babarin as described by Davenport [14]. Submandibular saliva was collected from sheep under general anaesthesia by aspiration from the papilla of the submandibular duct. At the end of the experiment, the animal was killed by an injection of sodium pentobarbitone.

Analysis of data

Values are expressed as means $\pm$ S.D. Unmatched data were analysed by the Kruskal-Wallis one-way analysis of variance. For the VIP-infusion experiments, the matched data were analysed using the Wilcoxon matched-pairs signed-ranks test.

RESULTS

Radioimmunoassay characteristics

When CA VI was radioiodinated and subjected to gel filtration on Sepharose 6B, two radioactive peaks were obtained. Only the first peak could be immunoprecipitated with excess CA VI antibody and this peak incorporated 20–40% of the total radioactivity used. The specific radioactivity of the tracer was estimated at 2–5 μ Ci $\cdot$ μ g of protein $^{-1}$. With an excess of CA VI antibody (ten times the concentration used in the radioimmunoassay), 93% of the total radioactivity was precipitated. When the tracer was run on an SDS/polyacrylamide gel and the dried gel autoradiographed, more than 98% of the ^{125} I radioactivity corresponded to the 45 kDa CA VI band on the gel (results not shown).

Fig. 1. Comparison of the typical standard curve for sheep CA VI and dilutions of a saliva sample

The percentage of antibody-bound radioactivity is plotted against the amount of standard CA VI added (○) or the volume (μl) of saliva sample added (●). The values are corrected for non-specific binding of the tracer.

A radioimmunoassay standard curve for sheep CA VI is shown in Fig. 1. Non-specific binding for the assay was $3.6 \pm 0.5\%$ ($n = 10$). The 50% displacement of bound tracer was obtained at approx. 3.5 ng of CA VI. The sensitivity of the assay, defined as 2 S.D. from the zero tube, was 75 pg/tube.

The intra-assay precision calculated from 12 measurements of a saliva sample in one assay was 3.0% (for a mean value of 4.2 ng). The inter-assay variation, determined in ten assays over 2 months, was 10.5% (for a mean value of 3.7 ng). Dilutions of a saliva sample in the assay gave a curve parallel with the standard curve (Fig. 1), indicating that there are no interfering substances present in saliva to invalidate the assay for saliva samples. The assayed values of the saliva samples were not affected by storage at -20°C over 3 months.

The antibody used in the assay showed no cross-reactivity with sheep CA II or CA III up to 1 μ g ($< 0.01\%$), the highest values tested. It also showed no cross-reactivity with human CA VI (0.1 μ g, $< 0.1\%$).

Table 1. Output of CA VI from the sheep parotid gland

Values are means $\pm$ S.D. Statistical significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Expt.	[CA VI] (μ g $\cdot$ ml $^{-1}$)	CA VI secretion rate (μ g $\cdot$ min $^{-1}$)	CA VI specific activity (μ g $\cdot$ mg of protein $^{-1}$)	Protein secretion rate (μ g $\cdot$ min $^{-1}$)
Normal conscious animal ($n = 42$)	5.61 ± 3.01	11.7 ± 7.8	79.4 ± 35.7	166 ± 83
During feeding ($n = 9$)	$33.0 \pm 19.0^{***}$	$124 \pm 100^{***}$	$118 \pm 27^{**}$	$1063 \pm 919^{**}$
Anaesthetized neurotomized animal ($n = 9$)	$2.87 \pm 1.46^*$	$0.62 \pm 0.40^{***}$	$18.1 \pm 8.0^{***}$	$38.4 \pm 19.3^{***}$

Table 2. Effect of stimulation of the neurotomed parotid gland on CA VI secretion in anaesthetized sheep

Values are given means $\pm$ S.D. Statistical significance: ** $P < 0.01$; *** $P < 0.001$.

†Stimulation for 1–4 min.

‡Stimulation for 30–60 min.

Expt.	[CA VI] ($\mu\text{g} \cdot \text{ml}^{-1}$)	CA VI secretion rate ($\mu\text{g} \cdot \text{min}^{-1}$)	CA VI specific activity ($\mu\text{g} \cdot \text{mg of protein}^{-1}$)	Protein secretion rate ($\mu\text{g} \cdot \text{min}^{-1}$)
Anaesthetized neurotomed (control) ($n = 9$)	2.87 ± 1.46	0.62 ± 0.40	18.1 ± 8.0	38.4 ± 19.3
Bethanechol-stimulated ($n = 8$)	$149 \pm 33^{***}$	$438 \pm 172^{***}$	$131 \pm 43^{***}$	$3743 \pm 1540^{***}$
Moussu-nerve-stimulated† ($n = 4$)	$176 \pm 127^{***}$	$634 \pm 330^{***}$	$152 \pm 138^{***}$	$5720 \pm 3921^{**}$
Prolonged Moussu-nerve stimulation‡ ($n = 4$)	$9.2 \pm 3.3^{**}$	$18.1 \pm 7.5^{**}$	$82 \pm 4.1^{**}$	$222 \pm 98^{**}$

CA VI concentrations in saliva

The mean CA VI concentration in 42 samples of parotid saliva obtained by cannulation of five normal conscious unstimulated sheep was $5.6 \pm 3.0 \mu\text{g} \cdot \text{ml}^{-1}$, with a range of 2.0 – $9.8 \mu\text{g} \cdot \text{ml}^{-1}$. In relation to total protein this was $79.4 \pm 35.7 \mu\text{g}$ of CA VI $\cdot$ mg of total protein $^{-1}$, with a range of 11 – $233 \mu\text{g} \cdot \text{mg}^{-1}$. The corresponding mean secretion rate was $11.7 \pm 7.8 \mu\text{g}$ of CA VI $\cdot$ min $^{-1}$. Submandibular saliva contained $6.9 \pm 2.1 \mu\text{g}$ of CA VI $\cdot$ ml $^{-1}$ ($n = 3$). Feeding stimulated the output of CA VI in saliva of normal animals about 10-fold, and these results are shown in Table 1. The rate of secretion of CA VI from the neurotomed parotid gland of anaesthetized sheep was lower than that from normal conscious sheep at $0.62 \pm 0.40 \mu\text{g} \cdot \text{min}^{-1}$ ($n = 9$) (Table 1). However, both bethanechol and electrical stimulation of Moussu's nerve supplying the parotid gland led to large increases in both salivary flow rate and concentration of CA VI. With continued stimulation of Moussu's nerve (30–60 min), the CA VI concentration and secretion rate and the protein-secretion rate fell significantly, but were still at levels significantly above the 'anaesthetized' control values. These results are summarized in Table 2. The CA VI content of the parotid gland subjected to prolonged stimulation of the Moussu nerve (30–60 min) fell from $6.3 \pm 2.7 \mu\text{g} \cdot \text{mg}$ of protein $^{-1}$ to $3.8 \pm 1.6 \mu\text{g} \cdot \text{mg}$ of protein $^{-1}$. Although this fall was not statistically significant (probably owing to the low number of samples; $n = 4$), there was a fall in the intensity of CA staining in histological sections of this tissue (Fig. 2).

VIP infusion alone had no significant effect on salivary flow rate (control: $0.26 \pm 0.06 \text{ ml} \cdot \text{min}^{-1}$, $n = 5$; VIP: $0.26 \pm 0.03 \text{ ml} \cdot \text{min}^{-1}$, $n = 5$), but caused a small increase in the total protein secretion rate (control: $21.0 \pm 8.9 \mu\text{g} \cdot \text{min}^{-1}$; VIP: $38.4 \pm 21.3 \mu\text{g} \cdot \text{min}^{-1}$; with a two-tailed P value < 0.05) and in the rate of secretion of CA VI (control: $0.40 \pm 0.17 \mu\text{g} \cdot \text{min}^{-1}$; VIP: $0.96 \pm 0.63 \mu\text{g} \cdot \text{min}^{-1}$; $P < 0.05$). VIP plus bethanechol had no consistent effect on the rate of CA VI secretion.

The only tissues of sheep found to have measurable concentrations of CA VI were the parotid gland ($6.4 \pm 3.8 \mu\text{g} \cdot \text{mg}^{-1}$, $n = 3$) and the submandibular salivary gland ($1.8 \pm 0.4 \mu\text{g} \cdot \text{mg}$ of protein $^{-1}$, $n = 3$). The cerebrospinal fluid, kidney, lung, adrenal, brain, skeletal muscle, liver, heart, pancreas and small intestine did not have measurable CA VI levels.

DISCUSSION

A sensitive and specific radioimmunoassay system has been developed to measure accurately and rapidly concentrations of

Fig. 2. CA activity in unfixed 10 μm frozen sections of sheep parotid gland before (a) and after (b) prolonged stimulation of the Moussu nerve supplying the gland

CA activity was detected by the method of Coquin-Carnot & Barbarin (as described by Davenport [14]), and the nuclei were counterstained with Mayer's haematoxylin for 30 s. The bar represents 100 μm .

CA VI in saliva and tissues. Routinely, 1 μl samples or less of saliva were used to measure CA VI. The antibody used did not show cross-reactivity with sheep CA II and CA III nor with human CA VI, although in Western analysis using denaturing gels this antibody did detect human CA VI and a protein from skeletal muscle, presumably CA III [4]. The epitope(s) recognized

by this antibody is apparently masked in the native state of the latter proteins.

CA VI in normal unstimulated sheep saliva at $5.6 \pm 3.0 \mu\text{g} \cdot \text{ml}^{-1}$ is one of the major protein components of saliva, constituting 8% of the total protein. Submandibular saliva has a similar concentration of CA VI, but the flow rate is lower (about $0.05 \text{ ml} \cdot \text{min}^{-1}$), giving a lower secretion rate of CA VI from the submandibular gland. This is reflected in the lower concentration of CA VI in the submandibular gland itself (about one-third of the concentration in the parotid) and is also reflected in the relative levels of CA VI mRNA in the two glands [4]. CA VI appears to be synthesized exclusively in these salivary glands, confirming our earlier findings using Western analysis to investigate tissue distribution [4].

Salivary flow rate and protein concentration are under the control of both sympathetic and parasympathetic pathways [12,15]. Feeding is known to stimulate both the flow rate and protein concentration, with the latter effect being partially blocked by the β -adrenergic-receptor-blocking agent propranolol [15]. In our study, saliva flow rate and total protein secretion increased with feeding, thus confirming earlier reports [15,16], with CA VI concentrations paralleling the pattern of protein secretion, being high initially with a tapering off over the ensuing 30 min. There was a significant increase in the specific activity of CA VI in the saliva during feeding (from 7.9% of total proteins to 11.8%), indicating a specific stimulation of CA VI release over and above that of the other proteins. A larger increase (20% of the total protein of saliva to 39%) in an unidentified 45 kDa protein has been observed previously in the sheep by other workers [16]. Presumably the protein is CA VI. The mechanism for this higher rate of CA VI secretion is not known. With the neurotomized sheep preparation, CA VI concentration, secretion rate and specific activity were all significantly lower than in normal conscious sheep. After stimulation by bethanechol, which is specifically muscarinic, there was a very large increase in these parameters. Flow rate increased 13-fold, protein secretion rate 100-fold, and CA VI secretion rate 706-fold compared with the anaesthetized controls. There was a corresponding significant rise in the specific activity of CA VI in the saliva. Electrical stimulation of Moussu's nerve, a parasympathetic nerve, gave similar large increases in these parameters.

Vasoactive intestinal polypeptide has been found in neurons of the sheep parotid gland [17], and there is evidence for the co-existence and co-release of VIP and acetylcholine from the parasympathetic nerves of the cat submandibular gland [18]. It has been reported [19] that VIP infusion into the parotid glands

of sheep causes an increase in salivary flow and protein concentration. Although these studies did not find an increase in salivary flow rate, there was a significant increase in both the protein and CA VI secretion rates. It has been observed (R. D. Wright, unpublished work) that VIP potentiates the secretory response to bethanechol stimulation, although no consistent effect was observed for CA VI secretion. However, it does appear that VIP could be involved in the secretion of CA VI. Further investigation is required to determine how the secretion of CA VI is controlled.

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REFERENCES

1. Tashian, R. E. (1989) *BioEssays* **10**, 186–192
2. Dodgson, S. J., Foster, R. E., II, Storey, B. T. & Mela, L. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5562–5566
3. Wistrand, P. J. (1984) *Ann. N. Y. Acad. Sci.* **429**, 195–206
4. Fernley, R. T., Darling, P. E., Aldred, P., Wright, R. D. & Coghlan, J. P. (1989) *Biochem. J.* **259**, 91–96
5. Fernley, R. T., Coghlan, J. P. & Wright, R. D. (1988) *Biochem. J.* **249**, 201–207
6. Fernley, R. T., Wright, R. D. & Coghlan, J. P. (1988) *Biochemistry* **27**, 2815–2820
7. Feldstein, J. B. & Silverman, D. N. (1984) *J. Biol. Chem.* **259**, 5447–5453
8. Murakami, H. & Sly, W. S. (1987) *J. Biol. Chem.* **262**, 1382–1388
9. Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–210
10. Bolton, A. E. & Hunter, W. M. (1973) *Biochem. J.* **133**, 529–539
11. Abraham, S. F., Coghlan, J. P., Denton, D. A., McDougall, J. G., Mouw, D. R. & Scoggins, B. A. (1976) *Q. J. Exp. Physiol.* **61**, 185–192
12. Coats, D. A., Denton, D. A., Goding, J. R. & Wright, R. D. (1956) *J. Physiol. (London)* **131**, 13–31
13. Blair-West, J. B., Fernley, R. T., Nelson, J. F., Wintour, E. M. & Wright, R. D. (1980) *J. Physiol. (London)* **299**, 29–44
14. Davenport, H. A. (1960) *Histological and Histochemical Techniques*, p. 369, W. B. Saunders, Philadelphia
15. Patterson, J., Brightling, P. & Titchen, D. A. (1982) *Q. J. Exp. Physiol.* **67**, 57–67
16. Voigt, P. L., Patterson, J. & Titchen, D. A. (1980) *Proc. Aust. Physiol. Pharmacol. Soc.* **11**, 38P
17. Wathuta, E. M. (1986) *Q. J. Exp. Physiol.* **71**, 615–631
18. Lundberg, J. M. & Hokfelt, T. (1983) *Trends NeuroSci.* **6**, 325–333
19. Reid, A. M. & Titchen, D. A. (1985) *Proc. Aust. Physiol. Pharmacol. Soc.* **16**, 27P

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