Preparation of Murine and Human Acute Adrenal Gland Slices.

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Introduction

The adrenal gland is a hormone gland present in many species. It has a peculiar embryological origin as it consists of an ectoderm-derived neuroendocrine medulla, secreting catecholamines (noradrenaline, adrenaline), and a mesoderm-derived cortex responsible for secreting steroid hormones into the blood stream.

Adrenal function can be investigated using a plethora of methods, but acute slice preparations provide a unique approach, providing good access for experimental interventions while maintaining cellular interactions within the tissue.

Our group uses acute adrenal gland preparations primarily to study the mechanisms of aldosterone synthesis in the outermost cell layer of the adrenal cortex, the zona glomerulosa. However, we also have made limited observations in the cortisol-producing zona fasciculata as well as in chromaffin cells of the adrenal medulla, suggesting that the following description works for the adrenal gland in general.

Most of our protocols have been established for studies of the mouse adrenal cortex[1,2] and the procedures described underneath mostly focus on this species. We have recently begun to work with human adrenal tissue samples and found that most aspects of our protocols translate well with notable exceptions stated below.

Procedures

The work on murine adrenal glands is mostly complicated by their small size (~1 mm in diameter). It is therefore crucial to work carefully to obtain optimal conditions for slicing on a vibratome. This can increase the duration of each step in the whole procedure. To avoid hypoxia, we carry out all work in bicarbonate-buffered salt solution that are constantly gassed with carbogen (95% O_2, 5% CO_2) to maintain oxygenation and pH buffering (base solution for murine samples (in mM): 102 NaCl, 26 $NaHCO_3$, 15 NaGluconate, 8 D-Glucose, 5 HEPES, 2 KCl, 2 KGluconate, 1 $MgCl_2$; Add 2 mM $CaCl_2$ only after setting the pH 7.4 to avoid the formation of insoluble $CaCO_3$).

An important difference in the work with murine or human tissue samples lies in the osmolality of the solutions as the adrenal cortex is an exquisite osmo-sensor. Mice have a higher, strain-dependent osmolality of ~315 mOsmol while the osmolality of human plasma centers around 290 mOsmol.

To facilitate embedding into agarose for slice cutting, it is strongly suggested to first create a clean preparation of the adrenal tissue. The adrenal glands are contained within a fibrotic capsule that is not easily torn apart and can be used to determine the border of the organ itself. Using small forceps and scissors under

a stereo microscope, remove the fatty and connective tissue attached to the outside of the capsule.

Due to their small size and to facilitate the cutting of rather thin sections, embedding of adrenal glands or tissue in agarose is crucial. A concentration of 4% of low-melting temperature agarose provides a good compromise between viscosity when heated and rigidity when cooled down.

We use multi-well plates (24-well for murine glands, up to 6-well for human adrenal samples) as a mold for embedding. One well is filled with an appropriate volume of heated (~37 °C) 4% agarose and the samples are carefully placed into the agarose. Before, the tissue samples should be blotted against a tissue to remove excess liquid which might dilute the agarose in the immediate vicinity of the sample, rendering it prone to breaking away from the agarose during cutting. Murine adrenal glands can also be mixed in with the agarose by slightly swirling them inside of the well before hardening. When using both adrenal glands of a mouse, it is possible to embed both organs into the same agarose well a few mm apart (**Fig.1**). Special care should be taken to have both glands settle at approximately the same height within the agarose block to speed up the slicing process.

After removal from the well (injecting solution underneath the agarose using a syringe will easily push out the block), the agarose block is glued to the stage using cyanoacrylate glue. Trimming cuts should be made as required. We typically cut a straight line perpendicular to the leading edge of the cutting blade, leaving approximately 5 mm of agarose in front of the tissue sample across the whole block. A similar cut can be made ~5 mm behind the organ to minimize the required duration of the cut (Fig. 1). If two organs are embedded within the same block, a separating cut can be made between the two. We typically only remove the now separated agarose between the sample and the blade while leaving the rest in place for stabilization (**Fig.1**).

The stage is then placed into the cutting tray of the vibratome (7000smz-2, Campden Instruments) filled with bicarbonate-buffered solution under constant oxygenation. We found no clear advantage of using lowered temperatures during this step, so everything is carried out at room temperature. The use of ceramic blades is highly recommended due to their increased stiffness and longevity compared to the often-used razor blades. Deflection of the blade in the z-direction must be minimized (we calibrate to ≤0.3 µm). Slices are now cut (frequency: 90 Hz, amplitude: 1.5 mm) with the thickness set according to experimental requirements. Because of the small size of murine adrenal glands, it is advisable to carefully approach the height of the organ embedded in the agarose. This can be achieved by first making faster trimming cuts at 500 µm thickness and then slowly reducing this setting to the desired final thickness. Such an approach also minimizes any backlash in the mechanism lifting the sample table. Once the correct height has been reached, we use a slow cutting speed of 0.1 mm/s within the agarose that is decreased down to 0.02 mm/s when cutting through the tissue itself to avoid tearing the sample from the agarose and to improve the integrity of the slice. Once satisfactory slices have been obtained, the "profile repeat mode" can be used to repeat the same cutting profile for 4-6 times.

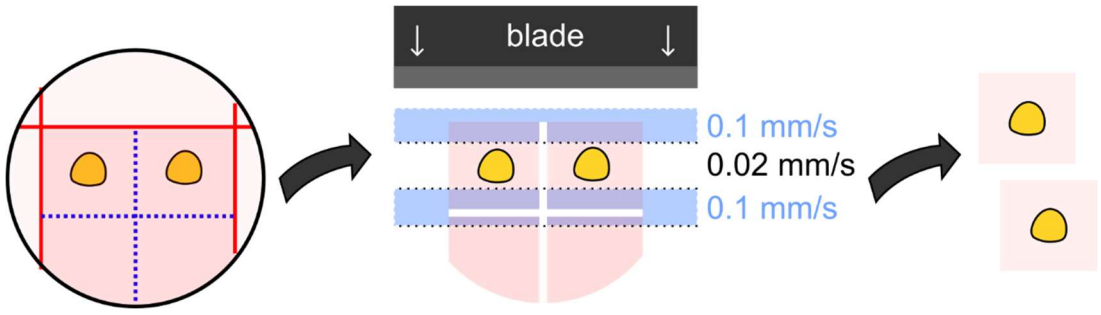

Fig.1. Schematic drawing of the steps required to generate acute murine adrenal slices. Whole adrenal glands (orange) are placed in the center of a 24-Well (black outline). The well is then filled with 4% agarose and put at 4 °C until hardened. Following removal from the well, trimming cuts are made with a scalpel. Cuts are either made through the whole agarose block (red lines) or only to slightly below the level of the adrenal glands within the block (blue dotted lines). The block is then glued to the sample holder and the vibratome is used to cut slices at speeds of 0.1 mm/s within the agarose and 0.02 mm/s while cutting through the tissue, resulting in two adrenal slice per cutting cycle.

The adrenal cortex is rather opaque with thin slices (60-80 µm) having the best optical transmissibility allowing for easier identification of single cells, for example in patch clamp studies, at the expense of reducing yield. Medium slices (120-160 µm) are a good compromise of providing good yield, reasonable optical

transmissibility for fluorescence experiments, in particular calcium imaging, and mechanical stability. Thicker slices (≥200 µm) are suited for long-term incubations, purification of nucleotides and proteins as well as observations of hormone production. They provide a low yield overall due to the small number of slices being generated from a single adrenal gland but excellent mechanical stability. For human samples, 200 µm thick slices are a more reasonable overall choice because of the larger organ size.

The adrenal medulla with its chromaffin cells is more transparent and single cells can be readily identified even within thicker sections. Earlier protocols describe the use of 200-300 µm thick slices of rat adrenal glands for electrophysiological investigations[3,4] and we also have observed calcium signals at single cell level using this thickness.

Following cutting, slices can be stored in carbogen gassed solutions for up to 8 hours before a drop in the quality and reproducibility of results becomes apparent. It is possible to maintain mouse adrenal tissue slices in an interface culture for at least one more day and human slices can be maintained for 3 days typical calcium transients still being observable. However, we have not made any systematic evaluation into whether these signals are fully comparable to those measured on the first day.

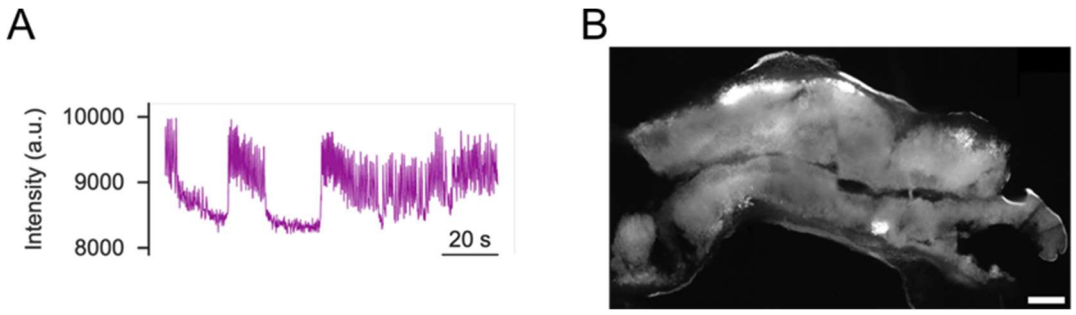

Fig.2. (A) Example of the calcium signals from a single zona glomerulosa cell within a murine adrenal gland slices. The slice was stained with Calbryte 520 AM for 1 hour prior to imaging. Calcium signals are stimulated by exposure to 1 nM angiotensin II and 4 mM K+. (B) Example of a 150 µm thick human adrenal gland slice fixed with -20 °C cold methanol for 20 minutes and stained with an antibody against the human aldosterone synthase (CYP11B2; white areas). Scale bar: 0.5 mm.

Specific examples

We have used the generated acute adrenal gland slices with great success to record the calcium signaling (Fig. 2A) that links physiological stimulation of the zona glomerulosa with angiotensin II or potassium to aldosterone synthesis. For this, we rely on the staining of acute adrenal slice preparations with organic, calcium-sensitive dyes such as Calbryte 520 AM1 or Fura-22. It is also possible to combine this imaging modality with pharmacological experiments as we have done in the past to dissect the contribution of T- and L-type voltage-gated calcium channels to the calcium influx into the zona glomerulosa[5]. The same technique also works on human adrenal gland slices, an approach we're currently working on. Other groups have also used slices prepared by a similar method from mice expressing a genetically encoded calcium sensor[6].

Acute adrenal slice preparations have also been used by some groups in patch clamp experiments of cells from the adrenal cortex[7] or medulla[4].

It is also possible to use these rather thick slice preparations for antibody stainings. With a prolonged incubation, we were able to reliably identify aldosterone producing (micro-)nodules in human adrenal gland slice preparations (Fig. 2B).

Additionally, we've also successfully used murine adrenal gland slices to measure aldosterone and corticosterone production in the supernatant of slice incubations over several hours. Also, while slices stemming from vibratome sectioning are thicker than from other techniques, they can also be used for immunofluorescence, something that we now regularly use to identify aldosterone-producing cells from human adrenal slice preparations.

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