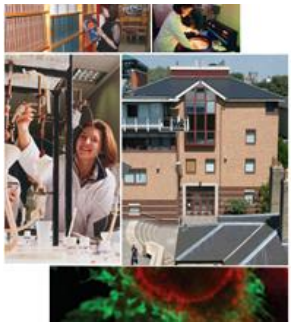
UNIVERSITY OF CAMBRIDGE



Ca2+ Taylor Lab signalling

PHARMACOLOGY

Master Class

Saturday 17 March 2012

Department of Pharmacology
University of Cambridge

As pharmacologists, we are concerned with understanding how drugs work. These drugs might be the medicines used to treat disease, recreational drugs like caffeine, drugs of abuse like heroin, or they might be environmental pollutants. The pharmacologist aims to understand the common principles behind these drug actions and to apply those principles to the development of better drugs.

Today we will focus on smooth muscle because it is a major target of many important drugs used, for example, to treat asthma and high blood pressure, and to induce labour.

Programme

10.30	Arrival and registration
10.40	Introduction
11-1	Practical session 1 (muscle contraction or Ca^{2+} signals)
1-1.30	Lunch (in the Tea Room on level 4)
1.30-3.30	Practical session 2 (Ca^{2+} signals or muscle contraction)
3.30-4.30	Optional tours of Pembroke College

If you need to make arrangements for transport home, you should be free for collection at the Department of Pharmacology from about 4.45pm if you take the tour, 3.45pm otherwise.

Funding for the facilities used today was provided by:

BBSRC

wellcome trust

MRC Medical Research Council

Muscle

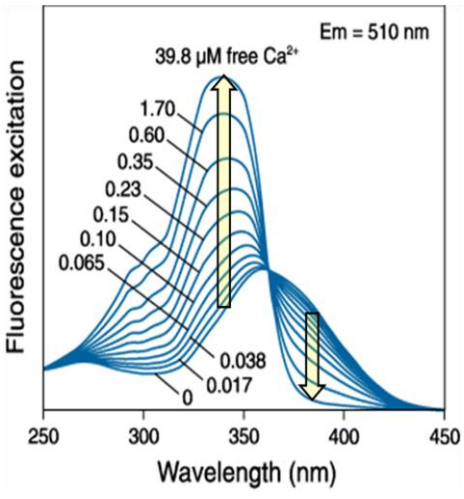
Striated muscles, the muscle that forms the heart and the muscles that allow voluntary movements, derive their name from the regular arrays of contractile fibrils within each muscle cell; these are adapted to allow rapid and usually brief contractions. Their activities allow the heart to beat regularly ($2x10^9$ times in an average human lifespan) and they allow you to run, jump and play the piano.

Smooth muscles are different: the fibrils do not form regular arrays and nor are the muscles under voluntary control. These muscles are important in regulating such activities as the aperture of the iris of your eye (controlling the amount of light that can enter), the peristaltic activity of the intestines (propelling food along the gut), contractions of the uterus during childbirth, and the width of both the small airways in the lung (which become too narrow during an asthma attack) and of blood vessels (thereby controlling blood pressure and local blood flow). Many of these smooth muscles are important targets of drugs used clinically to treat high blood pressure, erectile dysfunction (Viagra), asthma (Ventolin), premature labour or induction of labour, and diarrhoea (Immodium).

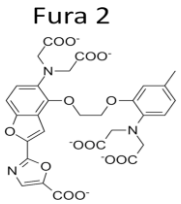
Although striated and smooth muscles differ in their gross appearance and physiological roles, both types of muscle contract when the concentration of free Ca^{2+} ions increases in the cytoplasm of the muscle cell. In all muscles, although the details differ between smooth and striated muscle, Ca^{2+} encourages the myosin heads that protrude from the thick muscle filaments to crawl along the thin actin filaments. This movement, fuelled by the energy provided by hydrolysis of ATP, allows the two filaments to slide between each other causing the muscle to shorten. One component of today's practical will use a sensitive force transducer to measure the contraction of smooth muscle within a short piece of gut.

Ca^{2+} signals

All cells work hard to extrude Ca^{2+} from their cytoplasm, and this leaves them with steep Ca^{2+} concentration gradients across both the membrane that surrounds the cell (the plasma membrane) and across the membranes that form the endoplasmic reticulum (ER) within the cell. By transiently opening channels within these membranes, cells allow Ca^{2+} rapidly to flow into the cell and thereby to regulate many different cellular activities, including muscle contraction. Many extracellular stimuli, like hormones or neurotransmitters, exert their effects on their target cells by first binding to a receptor in the plasma membrane and thereby initiating a cascade of events that culminates in opening of Ca^{2+}-permeable channels within either the ER or the plasma membrane. The second component of today's practical will allow you to measure changes in the Ca^{2+} concentration of cultured cells stimulated with one of the neurotransmitters that causes muscle to contract.



These experiments are made possible by the work of Roger Tsien while he was in Cambridge and who received the Nobel Prize in Chemistry in 2008. He developed sensitive fluorescent Ca^{2+} indicators, including the fura 2 that you will use today. These indicators can be easily loaded into living cells and used to measure changes in their free cytoplasmic Ca^{2+} concentration. An important feature of fura 2, which you will exploit today, is that when it binds Ca^{2+}, the light it emits when excited at a short wavelength (340 nm) increases, while the light emitted following excitation by light with a longer wavelength (380 nm) decreases (see Figure). By loading cells with fura 2 and then monitoring their fluorescence (510 nm) while rapidly switching between exciting light with shorter wavelengths (340 and 380 nm), we can detect changes in cytoplasmic free Ca^{2+} concentration.



Practical sessions
You will need to divide yourselves into groups of 3 (for contraction measurements) and 3 or 4 (for Ca^{2+} measurements). Half the group will do each practical before you swap places after lunch.

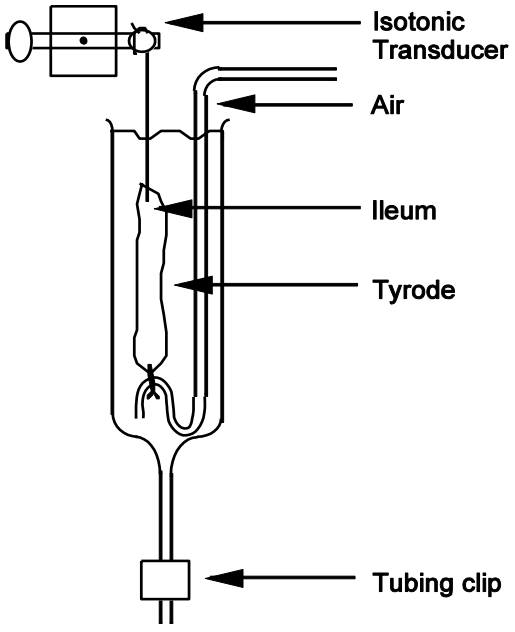
The equipment you will use is expensive (>£150,000 for the F4500 instruments used to measure Ca^{2+}) and some of the reagents you will use are toxic. You must read the risk assessments posted alongside each experimental rig before beginning work. **Note the need to wear gloves and an apron**. If you have a lab coat of your own, please bring it, but spares will be available in the lab.

Practical 1: Contraction of ileum
Isolated organs are often used by pharmacologists to examine the actions of drugs. Intestine is particularly useful because it contains smooth muscle and the neurotransmitters released by gut neurons are also found in brain. In this session you will measure contractions of the longitudinal smooth muscle of the ileum evoked by addition of two endogenous neurotransmitters, acetylcholine and histamine. These drugs stimulate the muscle to contract – pharmacologists refer to such drugs as ***agonists***, because they ***activate*** the receptors to which they bind. Other drugs can bind to the same receptors, but fail to activate them; such drugs are described as ***antagonists***: they bind to receptors and thereby occlude access to the drugs that could otherwise activate them. Both classes of drugs are clinically important: mepyramine, for example, is an antagonist of histamine receptors and thereby blocks the ability of histamine to trigger the itching and other symptoms of hay fever. In this practical, you will examine the interactions between four drugs that interact with two receptors expressed in the longitudinal muscle of the ileum.

	Acetylcholine receptor	Histamine receptor
Agonist	Acetylcholine	Histamine
Antagonist	Benzilylcholine	Mepyramine

Your aim is to establish whether histamine and acetylcholine interact with the same or different receptors.

A small section of ileum, bathed in medium (Tyrode's solution) designed to mimic extracellular fluids and bubbled with oxygen has been mounted in an organ bath and connected to a force transducer to allow you to record contractile activity (see Figure). Adjust the amount of Tyrode in the centre-piece 10ml (~1cm from the top). Start recording and leave the preparation for a few min. Add acetylcholine (1-2 x 10^{-7}M final concentration). Insert the tip just below the surface and add the dose smartly, but not directly at the tissue. After 30s, wash the drug out by emptying the centre-piece and then refilling it. Do not repeat immediately, because you must allow time for the fresh Tyrode in the warming coil to reach the bath temperature. The suggested doses are only a guide because individual tissues do vary. The demonstrators will advise on how to adjust the dose to obtain a suitable response. Repeat the stimulation, using a strict time schedule (30s contact, and 2min from washout to next dose), until consistent responses are obtained. Repeat using histamine (try 5 x 10^{-7}M first).



After two tests each of histamine and acetylcholine, add 10^{-7}M benzilylcholine immediately after washing out the previous drug. Then, following the usual timing, examine the effects of acetylcholine and histamine. Repeat the procedure using 10^{-7}M mepyramine as the antagonist. The demonstrators will advise on any need to adjust the concentration of the antagonists.

Discuss your results and their interpretation with your demonstrator.

Practical 2: Ca^{2+} measurements in HEK cells

Cultured cells also have their place in pharmacological analyses: they are simpler than organs, they are often less variable in their responses, they offer opportunities for biochemical and molecular biological analyses that are much more difficult with complex organs, and they reduce the need for use of animals. The human embryonic kidney (HEK) cells that you will use today are often used in research because they are derived from human tissue, they are easy to grow in culture, and they are amenable to many different biochemical and molecular genetic analyses. HEK cells express similar acetylcholine receptors to those expressed in the ileum (they are described as muscarinic acetylcholine receptors); activation of these receptors gives rise to an increase in cytoplasmic Ca^{2+} concentration.

HEK cells have been grown for you on a thin sheet of glass, and a few hours before you arrived they were loaded with the Ca^{2+} indicator, fura 2. The sheet of cells is now mounted in a small chamber filled with a saline (HBS, hepes-buffered saline) designed to mimic the extracellular medium, and held within the fluorescence spectrometer that will allow you to record the light emitted from the fura 2. Tubing links the chamber to a pump that allows the cells to be continuously perfused with HBS, and for the perfusing medium to be changed rapidly. You will use carbamylcholine, which is a more stable version of the naturally occurring neurotransmitter acetylcholine, to activate the acetylcholine receptors and so evoke increases in cytoplasmic Ca^{2+} concentration.

Your aim is to establish whether the Ca^{2+} signals evoked by activation of acetylcholine receptors are generated by release of Ca^{2+} from stores within the cell or by Ca^{2+} entering across the plasma membrane.

You will be provided with HBS, Ca^{2+}-free HBS and a stock solution of carbamylcholine. Your demonstrators will advise on the most appropriate concentrations to use.

To allow reliable comparisons between successive stimuli, it is important to adopt a standard stimulus regime. Try stimulating with carbamylcholine for 5min before washing it out and allowing a recovery period of 5min before you stimulate again.

Discuss your results and their interpretation with your demonstrator.