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Mini-Review

Electrophysiological methods for drug assessment

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Background: Drug assessment is an important step to determine the effectiveness and safety of each compound. Any candidate substances are repeatedly tested to examine the activity and working mechanism. Drugs produce various effects through interaction with ionotropic receptors. Electrophysiological methods are used to detect such effects by monitoring the drug-ionotropic receptor interaction. Researchers are now focusing to develop simple and quick methods for drug assessment.
Objective: This mini-review presents current drug assessment using electrophysiological methods, and clarifies various problems involved in the assessment.

Keywords: Drug assessment, electrophysiological method, extracellular recording, ionotropic receptor, patch-clamp, voltage-clamp.

"Drug assessment" is an important step to determine the safety and efficacy of candidate substances. According to recent reports [1, 2], an average pre-tax cost for a new drug to bring it into clinical service is estimated at approximately $800 million. The current process of drug assessment is illustrated in **Fig. 1.** The drug assessment method is expected to be: 1) quick, 2) sensitive, 3) precise, 4) low-cost, 5) low-invasive, 6) using a low-dose sample, and 7) of high-throughput form [3]. Existing methods for drug assessment cannot meet all of these requirements.

This mini-review presents current drug assessment using electrophysiological methods, and clarifies various problems involved in the assessment.

Receptors in drug activity mechanism

The concept of interaction between drug and receptor was proposed by J.N. Langley in 1878 [4]. He found that when one drug had a facilitatory effect on salivary secretion in cats, there was another drug having an inhibitory effect. These two drugs might act on a specific substance. This is now called "receptor".

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A receptor is bound to low-molecular compound or peptides in cells, and transmits signals inside the cell. In general, drugs combine with receptor, activating (or not activating) function of the receptor. An original molecule that binds and activates receptors is called "ligand". On the other hand, drugs that bind and activate receptors are called "agonist". Drugs that bind but do not activate any receptors are called "antagonist". For example, morphine is a ligand of the morphine receptor [5]. Low-dose nicotine is an agonist of the acetylcholine receptor, but high-dose nicotine is an antagonist of the acetylcholine receptor [6].

There are four types of receptors: 1) ionictropic receptors, 2) G-protein coupled receptors, 3) enzymatic activity receptors, 4) intracellular receptors. Receptors of type 1-3 exist on the cell membrane, while type 4 exists in the intracellular compartment. Recently, a number of reports have indicated that drugs have active mechanisms through binding with receptors [7].

Ionotropic receptors have been widely-studied. Many new drugs that act on ionotropic receptors have been developed [8]. We review the outline of interaction between ionotropic receptor and drug. This includes the function of ionotropic receptors, and conditions necessary for assessment of drugs that act on ionotropic receptors.

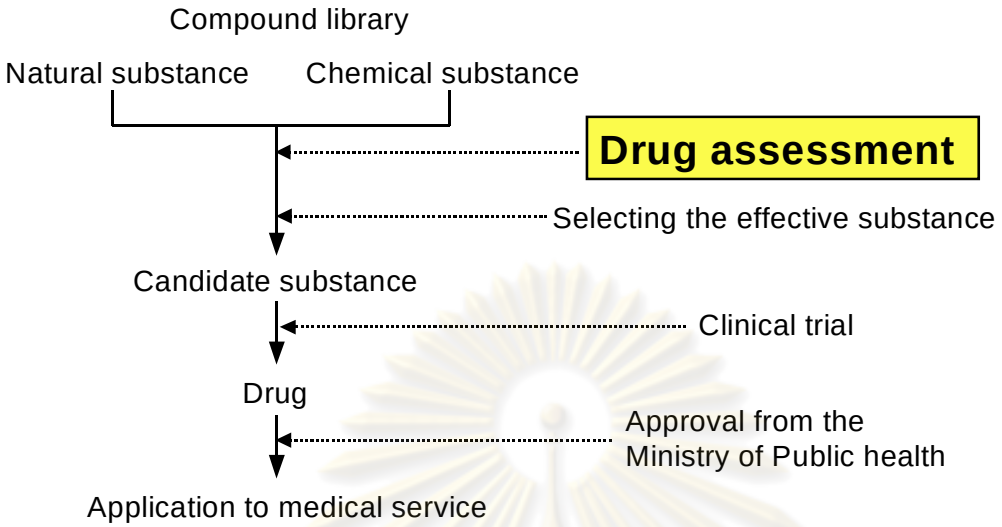

Fig. 1 Schema of the current process of drug assessment. In drug assessment, effective candidate substances are selected potentially by analyzing active mechanisms. When a candidate substance has a certain effect but has no side-effects, it is comprehensively examined for the safety. Only drugs which have passed these clinical tests, are applied for actual clinical service.

Ionotropic receptors and electrophysiological methods

In cells, charges across the membrane are separated. There are 5 most abundant ions on either side of the cell membrane. K^+ and organic ions are more concentrated inside the cell, while Na^+, Cl^- and Ca^{2+} are more concentrated outside the cell [9, 10]. This charge separation produces a difference in the electrical potential across the membrane that is called "membrane potential".

A majority of signaling transmissions in neuron or muscle cells are performed through changes of the membrane potential. A reduction of positive charges by the cellular influx, leading to less negativity of the membrane potential, is called "depolarization". On the other hand, an increase of positive charges by the cellular efflux, leading to more negative of the membrane potential, is called "hyperpolarization". These compartmental fluxes are controlled by ionotropic receptors (referred to Ligand-gated ion channels) [11]. These receptors are a group of intrinsic transmembrane ion channels that are opened or closed in response to binding of a chemical messenger [12]. Most ionotropic receptors have two functional sites: 1) the permeation control site, modulating "open" or "shut" of channel (*gate*) and 2) selective site for selecting permeable ion (*filter*). These filters have three important properties: i) conducting ions, ii) recognizing and selecting specific ions, and iii) opening and closing in response to specific chemical signals [13].

The function of an ionotropic receptor is greatly affected by agonist and antagonist drugs. For this reason, we believe that electrophysiological methods to assess the function of ionotropic receptors should be applicable for drug assessment. Electrophysiological methods for functional receptors are broadly classified into three categories: 1) voltage-clamp intracellular recording, 2) patch-clamp recording, and 3) extracellular recording [11]. Each recording has been widely applied to drug assessment. (For applied models, see [14]). In the followings, we present some applications of each measurement.

Voltage-clamp intracellular recording

The concept of voltage clamp was established by K.S. Cole and G. Marmont in 1940s [15, 16]. They discovered that the use of a two electrodes and a feedback circuit enable us to keep the membrane potential of cell at a set level [17]. According to this concept, researchers can measure the intracellular currents across the cell membrane by holding the membrane voltage at a set level. Current-voltage relationships of membrane channels have been studied using the voltage clamp by which the membrane voltage is manipulated independently of the ionic currents [11].

To evaluate ion flux across ionotropic receptors, researchers need to compensate any differences induced in cell's membrane potential [18]. The ionic causes of the action potential was elucidated in experiments using the voltage clamp by A. Hodgkin and A. Huxley in 1952 [19, 20].

The voltage clamp is composed of two electrodes: "voltage electrode" and "current electrode". Transmembrane voltage is recorded through a voltage electrode, relative to ground. A current electrode is used to pass current into the cell. In experiments, a voluntary voltage is set as "holding voltage" in which the voltage clamp uses negative feedback for maintaining the cell at this voltage level. An example of voltage-clamp intracellular recording is shown in **Fig. 2**. The electrodes are connected to an amplifier, which measures membrane voltage and feeds the signal into a feedback amplifier. Whenever the cell deviates from the holding voltage with the activity of ionotropic receptor, the operational amplifier generates an "error signal". The feedback circuit passes current into the cell to reduce the error signal to zero. Thus, the clamp circuit produces a current equal and opposite to the ionic current. This can be measured as "noise", which gives an accurate reproduction of the currents flowing across the ionotropic receptor [21].

For voltage-clamp intracellular recording, the two electrodes used are glass pipettes with very fine tips (<1 μm) so that it may clamp small cells [22]. For this reason, there are three kinds of weak points from the view-point of drug assessment. The first disadvantage is that microelectrodes are poor conductors. In fact, the electrode cannot sometimes pass current rapidly enough to compensate for ionotropic receptor's current. When the kinetics of the current (onset and offset) is very fast, the clamp cannot follow it faithfully. The second disadvantage involves "space clamp" issues. Microelectrodes can provide only a spatial point-source of current. Its effect is not uniform over different parts of an irregularly shaped cell. The third disadvantage is that the electrode is invasive to the cell. In experiments, microelectrodes are set directly on the cell, which gives odds against cell surviving.

Patch-clamp recording

This method was developed by E. Neher and B. Sakmann in late 1970s and early 1980s [23, 24], and is now used for recording the function of ionotropic receptor [11].

Patch-clamp recording is a refinement of the voltage-clamp intracellular recording. Traditionally, the patch clamp has used a glass pipette with an open tip (diameter~1 μm) that is known "patch-clamp

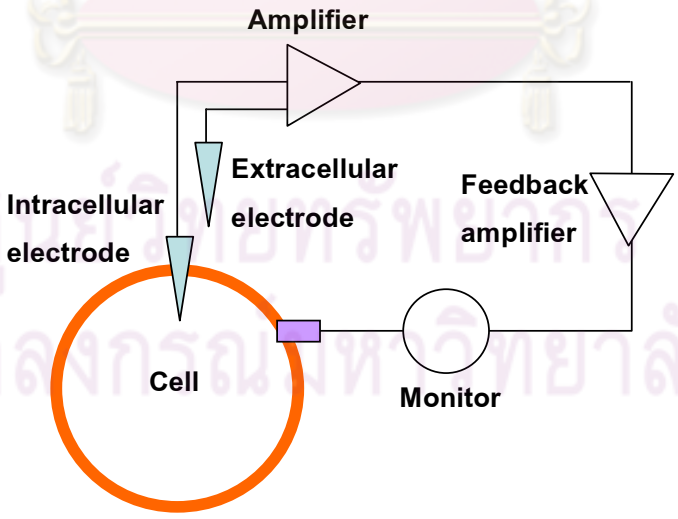

Fig. 2 Schema of voltage-clamp intracellular recording. The voltage clamp intracellular recording operates by negative feedback. The amplifier measures membrane voltage by intracellular and extracellular electrode. Output of amplifier is sent to the feedback amplifier; this subtracts the membrane voltage from the holding voltage. This signal is amplified and output is sent into the cell via the current electrode.

electrode" [25]. Its tip form is of a smooth surfaced circle, and distinct from the "sharp microelectrode" to be used in traditional voltage-clamp intracellular recordings. The pipette is filled with various solutions (dependent on specific techniques used). In whole cell recordings, for example, researchers use a solution to approximate the intracellular fluid. A metal electrode, contacting with this solution, conducts the electrical changes to a voltage clamp amplifier. Researchers can change the composition of the solution or add drugs to study the ionotropic receptors under different conditions. The patch clamp electrode is pressed against cell membrane. Suction is applied inside the electrode so that the cell membrane may be pulled inside the electrode tip. This causes the cell to form a tight seal between the electrode and the cell membrane. The electrical resistance of the seal is around one gigaohm. the so-called "gigaohm seal".

The patch clamp recording uses a single electrode to clamp a cell. The voltage can be kept constant by observing changes in current. Alternatively, the current constant can be kept constant by clamping the cell and observing changes in membrane potential. Thus, patch-clamp recording can record the changes of membrane currents and potentials which reflect the function of ionotropic receptors [26]. **Figure 3** shows several variations of the patch-clamp recording. These applications depend on what researchers want to study. In the following, we briefly describe the merit and demerit of each method.

Cell-attached patch. The electrode remains sealed to the patch of the membrane. This allows to record currents through ionotropic receptors in the patch of membrane [23]. Ionotropic receptors are activated by action of drug molecules. For this reason, the drug is usually included in the pipette solution. Ionotropic receptors are also activated through changes in potential across the membrane. The receptors can be clamped at different levels of membrane potential using the same patch. Researchers can establish this result of graded channel activation and a proper current-voltage curve using one patch only. Since the resulting channel activity can be attributed to the drug effect, researcher cannot change the drug concentration. The method is thus limited to one point

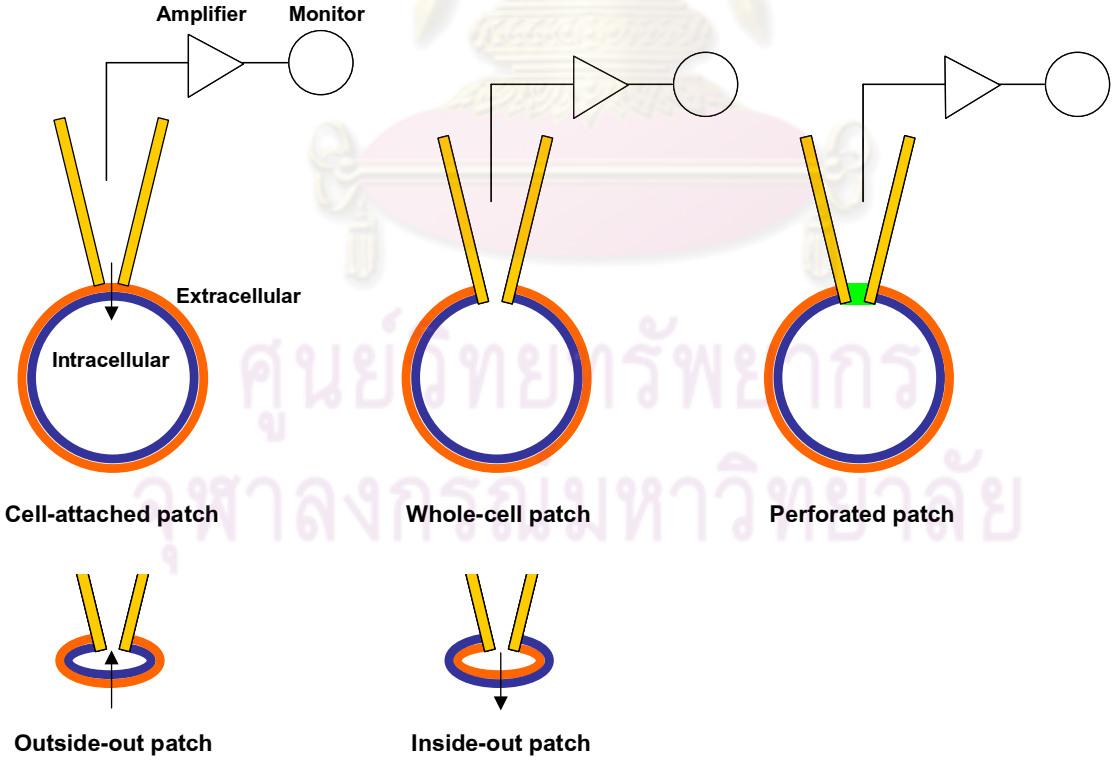

Fig. 3 Various applications of patch-clamp recording. The inside-out and outside-out methods are named "excised patch" because the patch is excised from the cell. Cell-attached and both excised patch methods are used to study the behavior of ion channels on the section of membrane attached to the electrode. Whole-cell and perforated patch allow researchers to study the electrical behavior of the entire cell.

in a dose response curve per one patch. Usually, the dose response is accomplished through several cells and patches [27].

Inside-out patch: After the gigaseal, the electrode is quickly withdrawn from the cell, thus ripping the patch of membrane off the cell, leaving the patch of membrane attached to the electrode, and exposing the intracellular surface of the membrane to the external solution [28]. This is useful when researchers wish to assess the drug effect which affects the inside of ionotropic receptors. For example, receptors that are activated by intracellular ligands like cGMP can then be studied through a range of ligand concentrations [29].

Whole-cell patch: The electrode is left in place, and more suction is applied to rupture the portion of cell membrane that is inside the electrode, thus providing access to the intracellular space of the cell [25]. The advantage of whole-cell patch clamp recording is that the larger opening at the tip of the patch clamp electrode provides lower resistance and better electrical access to the inside of the cell. Whole-cell patch has a place for drug assessment, because this method involves recording currents through multiple ionotropic receptors in a single cell at once. However, a disadvantage of this method is that the volume of the electrode is far larger than the cell, so the soluble contents of the cell will be slowly replaced by the pipette solution. This is referred to as the electrode "dialyzing" the cellular contents. Thus, any properties of the cell that depend on soluble intracellular contents will be altered. Generally speaking, there is a "washout period" at the beginning of a whole-cell recording, lasting approximately twenty minutes, when researcher must take measurements before the cell has been dialyzed [30]. This disadvantage makes whole-cell patch not suitable to assess drug effects that exert for a long stretch of time.

Outside-out patch: After the aforementioned whole cell patch is formed, the electrode is slowly withdrawn from the cell, allowing a bulb of membrane to "clip" out from the cell [25]. When the electrode is pulled far enough away, this clip will detach from the cell and reform as a ball of membrane on the end of the electrode, with the outside of the membrane being the surface of the ball. Outside-out patching gives researchers the opportunity to examine the properties of an ion channel when it is protected from the outside environment, but not in contact with its usual environment. In this conformation, researchers can perfuse the same patch with different solution of drugs, and if the ionotropic receptor is activated from the extracellular solution, a dose-response curve of drugs can be studied [31]. Single channel recordings are possible in this conformation if the clip of membrane is small enough [32]. This is the distinct advantage the outside-out patch variation possesses relative to the cell-attached method when applying to drug assessment. However, it is more difficult to accomplish outside-out patch, as more steps are involved in the patching process.

Perforated patch: This method was developed to resolve the problem of dialyzing in whole-cell patch by R. Horn and A. Marty [33]. This method is a variation of whole-cell recording. The researcher forms the gigaohm seal, adds a new solution to the electrode containing small amounts of an antibiotic, such as Amphothericin-B [33], Gramicidin [34] or β-escin [35], into the electrode solution to punch small perforations on the bit of membrane attached to the electrode. This has the advantage of preventing the dialysis of the cell that occurs in whole cell recordings, but also has several disadvantages. First, the access resistance is higher (access resistance is the sum of the electrode resistance and the resistance at the electrode-cell junction). This will not only decrease current resolution, but also increase recording noise and magnify any series resistance error. Second, it can take a significant amount of time (ten-thirty minutes) for the antibiotic to perforate the membrane. Third, the membrane under the electrode tip is weakened by the perforations formed by the antibiotic, and tends to rupture. When the patch ruptures, the recording is essentially in whole-cell mode, except with antibiotic inside the cell. All of these problems tend to limit the time-length of drug assessments, and so this method is most appropriate for short-duration drug assessments of about an hour [36].

Extracellular recording

The extracellular recording is a method to record extracellular field potential, that is an electrical potential (produced by cells and located outside of the cell). Many electrophysiological studies have investigated these potentials by using extracellular microelectrodes [37-39]. In experiments, the

extracellular field potential is detected as an electrical potential. Its source and component is often ambiguous, which makes its interpretation difficult. Individual cells may produce the change of potential through ionotropic receptors, which can be seen as peak of potential. For individual cells, the time-change of the extracellular potential is, in theory, inversely proportional to the transmembrane current generated by ionotropic receptors. In practice, however, this time-change is much complicated due to both complex morphology of cells and overlapped contributions from adjacent cells. The contributions from neighbouring cells are a disadvantage point of this method.

Recently, planar multi-microelectrodes array has been developed by H. Oka et al [40]. This system is dintrinct from traditional extracellular recording. The microelectrode arrays are microelectrode plates (50x50 μm) made from platinum black. The electrodes are sensitive to changes of potential, which enable us to assess drugs on single cell at high resolution **(Fig. 4)**.

This system can be applied to high-throughput drug assessment [41, 42]. However, there are several problems for application of extracellular recording, such as multi-microelectrodes array to drug assessment. First, extracellular recording is an indirect recording method. In fact, the change of potential around the electrode is an indicator, while voltage-clamp intracellular recording or patch-clamp recording are direct methods. Accordingly, it is necessary to perform the control experiment, together with test experiment. Second, ionic changes in extracellular potential are small, compared to patch-clamp recording. This is a problem for application to drug assessment in cell cultured experiments, which highlights the need for signal amplification, such as method for overexpressing ionotropic receptors on cell membrane [43].

Conclusion

Ionotropic receptor is a molecule to transmit information into the cell. Since P. Ehrlich [44], much attention has been paid to the function of ionotropic receptor from the aspect of drug assessment. If we can assess the interactions between drugs and ionotropic receptors accurately, we can identify the activity and working mechanisms of a number of drugs. A few electrophysiological methods have been developed to record the function of ionotropic receptors. Several methods are applied for practical purposes, but current methods are still very expensive and advanced techniques are required. Research on electrophysiological methods is promising for future drug assessment.

The author has no conflict of interest to declare.

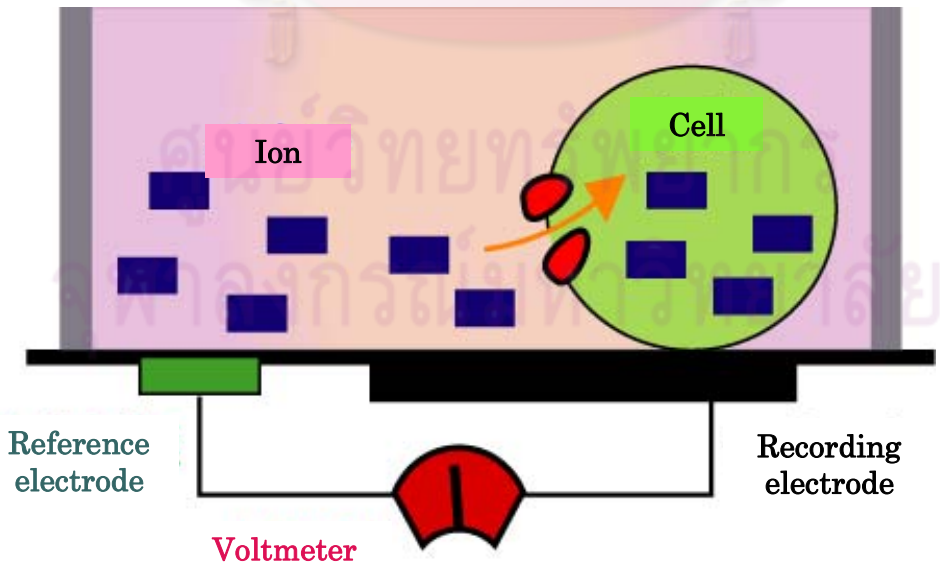

Fig. 4 Extracellular recording using a microelectrode plate. The change of potential around the recording electrode is recorded as an extracellular potential, which is subtracted by the potential around reference electrode.

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