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Title of Thesis: "Differential Expression of Inward and Outward Potassium Currents in the Macrophage-like Cell Line J774.1"

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Doctor of Philosophy Degree
April 2, 1985

Thesis and Abstract Approved:

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Committee Member	2 Apr 85 Date
Committee Member	2 Apr 85 Date
Committee Member	April 2, 1985 Date
Committee Member	2APR85 Date

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
APR 1985	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Differential Expression of Inward and Outward Potassium Currents in the Macrophage-1ike Cell Line J774.1	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Sciences Bethesda, MD 20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**150**	

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ABSTRACT

The electrophysiological properties of the J774.1 macrophage-like cell line were studied at 25 ^{o}C. Both conventional intracellular microelectrode and whole-cell patch microelectrode techniques were used. Long-term adherent (>24 hours) cultures investigated with intracellular electrodes exhibited a broad distribution of resting membrane potentials and membrane resistances (RMP = -50 +/- 3.0 mV, R_m = 117 +/- 13 Mohm, Mean +/- SEM, n = 55). Most cells with hyperpolarized RMP's exhibited inward rectification.

Cells studied with the whole-cell patch clamp technique under voltage clamp exhibited a narrow distribution of RMP's and R_m's (RMP = -77 +/- .78 mV, n = 37; R_m = 1,000 +/- 13 Mohm, n = 22, Mean +/- SEM). It was concluded that the increased breadth of the distributions obtained with intracellular electrodes was due to electrode penetration damage.

The expression of voltage-dependent conductances was studied as cells were transfered from suspension to adherent culture. Up to 1 hour after plating cells had essentially linear current/voltage relationships. Beyond 1 hour, a prominent outward rectifying current was evident. The fraction of cells exhibiting this current decreased with time in adherent culture, reaching zero after 12 hours. An inward rectifying current typically developed 2 to 4 hours after plating and was exhibited by all long-term adherent cells.

The inward rectifying current activated at potentials negative to -50 mV and inactivated with time at potentials negative to -110 mV; peak conductance was proportional to the square root of external potassium. Inward rectifying current was blocked by external barium or cesium.

The outward rectifying current activated at voltages positive to -40 mV and inactivated completely with time. The instantaneous current's reversal potential depolarized 52 mV per tenfold increase in external potassium. Outward rectifying current was blocked by compounds which block outward potassium currents in macrophages and lymphocytes.

Some characteristics of both currents changed during the first 10 minutes of a whole-cell patch recording but were stable thereafter. The outward current activated at more positive potentials after stabilization and peak conductance sometimes increased. In contrast, the voltage dependence of the inward current did not shift but peak conductance often decreased.

This work describes the ontogeny, voltage dependence and kinetics of potassium permeability in J774.1 cells. Such data provide a baseline for subsequent investigations of the role of voltage-dependent ion conductances in macrophage function.

DIFFERENTIAL EXPRESSION OF INWARD & OUTWARD POTASSIUM CURRENTS IN THE MACROPHAGE-LIKE CELL LINE J774.1

by

Paul Andrew Sheehy

Dissertation submitted to the
Faculty of the Department of Physiology
Graduate Program of the Uniformed Services University of
the Health Sciences in partial fufillment of the
requirements for the degree of
Doctor of Philosophy 1985

ACKNOWLEDGEMENT

I would like to take this opportunity to restate the cliche that this work would not have been possible without the support of family and friends. In point of fact, I think that I have been particularly fortunate in this regard. The contribution rendered by the advice and encouragement of others has been immeasurable but essential. Among the many who have endured my mood swings and scabrous outlook, three deserve particular mention:

Spencer Green-without whom I would never have had cultures to study.

Dr. David Livengood-who demonstrated extraordinary patience during my efforts to understand electricity and electrophysiology. But above all, my advisor.

Dr. Elaine Gallin-who has created an environment in which it is a genuine pleasure to do good work. She represents the highest standards of Science. Moreover, she deserves particular credit for her persistence at the sisyphean task of instilling such standards in me.

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INTRODUCTION

THE MACROPHAGE

Appreciation of the macrophage's role in both host defenses and immunology has grown substantially in recent years. Macrophages were conventionally viewed solely as scavengers that recycle senescent erythrocytes or remove debris from sites of injury (Copenhaver, Kelly & Wood, 1978). As the science of immunology has developed, it has been recognized that the macrophage is the central regulatory cell of the immune response (Rosenthal, 1980). Induction of antigen-driven T-lymphocyte proliferation requires the physical association of the T cell with a macrophage which presents the processed antigen (Rosenthal & Shevach, 1973). Genetic restiction of the immune response (self/non-self identification) is expressed through receptors on the macrophage's surface (Shevach & Rosenthal, 1973; Clark & Shevach, 1982). Macrophages augment secondary immune responses by receptor-mediated phagocytosis of opsonized particles (through receptors for immunoglobulin or complement molecules) and by secretion of hydrolytic enzymes and highly reactive oxygen metabolites. Finally, macrophages perform an essential role in the termination of the immune response (Allison, 1978).

Macrophages are characterized by a series of differentiation steps. All macrophages are ultimately derived from stem cells located in the bone marrow (Virolainen, 1968; Bainton & Golde, 1978). Progeny of

these stem cells develop over the course of a few days into monocytes which are released into the bloodstream. Monocytes remain in the circulation for up to a day and then enter tissues of the body where they mature into macrophages, surviving for several months or more. Macrophages are widely distributed throughout the body and are often specialized according to tissue type. In addition, macrophages within a given tissue can exist in different states of activation, referred to as resting, stimulated and activated (Rosenstreich, 1981). In the absence of inflammatory stimuli, a macrophage is said to be resting and is similar to its antecedent, the peripheral blood monocyte. A resting macrophage is transformed into a stimulated macrophage by stimuli such as endotoxin or by chronic inflammation produced by injection of specific irritants such as thioglycollate. These cells exhibit increases in spreading, receptor-mediated phagocytosis and secretion of superoxide anion and plasminogen activator. Subsequent exposure to factors released by stimulated lymphocytes will convert a stimulated macrophage to an activated cell able to kill tumor cells and intracellular parasites. Properties that are common to and used to identify macrophages, regardless of their state of activation, are Fc-mediated phagocytosis, expression of a non-specific esterase enzyme and adherence to glass substrates.

THE ROLE OF IONS IN MACROPHAGE FUNCTION

The macrophage is of particular interest to cell physiologists because, as a cell type, macrophages subserve a wide variety of functions in the host (Rosenstreich, 1981; Nathan, Murray & Cohn, 1980). These functions can be sorted into four broad categories: 1) ingestion and

degradation of all types of foreign material, 2) initiation and enhancement of lymphocyte activation, 3) direct destruction of tumor cells (Schultz, 1980) and certain microorganisms, and 4) suppression of the immune response (Allison, 1978). These functions employ cellular activities which include chemotaxis, both receptor-mediated and non-specific phagocytosis, the generation of reactive oxygen metabolites and the synthesis and secretion of a wide variety of biologically active compounds (a general overview of these activities is provided by van Furth (1980)). Many, if not most, of the mechanisms underlying these processes have requirements for specific ions. Therefore, changing ionic fluxes may well play a role in the initiation or regulation of macrophage function.

Secretion, phagocytosis and chemotaxis are all examples of cellular events which entail different aspects of cellular motility, whether it is of intracellular granules, the membrane or of the cell itself. Interactions between actin and myosin in the peripheral cytoplasm of macrophages probably provide the force for these events and intracellular Ca^{2+} controls the directionality (Stendhal & Stossel, 1980). Sensitivity to intracellular ionized calcium is conferred by the calcium-dependent regulatory protein gelsolin (Yin & Stossel, 1981) which controls the transition between the globular and filamentous forms of actin (Stossel & Hartwig, 1976). These changes in intracellular Ca^{2+} can occur through the influx of extracellular Ca^{2+} into the cell (possibly through voltage-gated channels) or through the release of intracellular calcium stores.

Many cell types use extracellular calcium which enters the cell via voltage-dependent channels (Hagiwara & Byerly, 1981) to activate

calcium-dependent mechanisms. Since the state of the channel (open or closed) is determined by voltage, other ionic conductances which modulate membrane potential, indirectly modulate Ca^{+2} influx. Thus, non-calcium ion conductances can affect cell function even though the particular mechanism has no explicit requirement for the ion. Therefore, most investigations of the role of ions in cell function have concentrated upon calcium. On the other hand, control of intracellular Na^{+}, K^{+} and H^{+} seems to be involved in the control of protein synthesis and cell growth. Therefore, voltage-dependent conductances of such ions may be related to control of these phenomena in macrophages.

Chemotaxis is associated with membrane potential changes in macrophages and a related phagocytic cell, the neutrophil (Gallin & Gallin, 1977; Seligman, Gallin, Martin, Shain & Gallin, 1980). External divalent cations (both Ca^{2+} and Mg^{2+}) are necessary for optimal chemotaxis by neutrophils (Gallin & Rosenthal, 1974). The observation that the chemotactic deficit of macrophages in divalent cation-free media can be reversed by low doses of ionophore (X537A or A23187) suggested that the release of intracellular divalent cations is involved in chemotaxis (Wilkinson, 1975). Interestingly, the ability of macrophages to orient in response to an externally applied electric field was inhibited by external Mg^{2+} or EGTA (Orida & Feldman, 1982).

The relationship between cell motility and membrane potential has been more extensively studied in the unicellular organism Paramecium. The model system of unicellular movement developed in Paramecium may be generalizable to many other cell types. The membrane of the Paramecium displays many of the ion channels characteristic of nerve and muscle (reviewed by Eckert & Brehm, 1979). The activity of these

channels in the membrane of the Paramecium has been directly related to the control of ciliary motility. Depolarizing stimuli activate a transient inward Ca^{2+} current which is followed by a complex combination of outward K^+ currents (Oertel, Schein & Kung, 1977). As intracellular Ca^{2+} rises, the ciliary beating motion reverses (Machemer & Eckert, 1973) and thus the Paramecium changes direction. Calcium current then declines due to a combination of two mechanisms: 1) channel closing due to membrane repolarization and 2) channel inactivation due to elevated intracellular Ca^{2+}. The membrane repolarization is accelerated by the activation of K^+ conductances such as the delayed rectifier and the Ca^{2+}-activated potassium conductance. Some idea of the importance of K^+ currents in determining the response to stimulation can be gained from so-called pantophobic mutants. These sub-lines are deficient in specific K^+ conductances and show exaggerated responses to stimulation (Saimi, Hinrichsen, Forte, & Kung, 1983).

A substantial component of phagocytosis by macrophages is sensitive to external calcium. Removal of external calcium decreases phagocytosis, whereas elevated calcium levels are stimulatory (Stossel, 1973). Agents which block mitochondrial uptake of intracellular calcium potentiate phagocytosis whereas D600, which blocks membrane calcium channels, inhibits phagocytosis (Kessler, Goodman & Carchman, 1980). Transient increases in intracellular ionized calcium during phagocytosis have been directly visualized using the flourescent calcium indicator Quin-2 (Young, Ko & Cohn, 1984). This last study also showed that phagocytosis in the absence of external calcium is nonetheless accompanied by a transient increase in intracellular ionized calcium.

Stimulus-secretion coupling is not as well understood in

leukocytes as in other cell types although secretion by leukocytes is accompanied by membrane potential changes. Both the macrophage and the basophil depolarize in response to secretagogues (Young, Unkeless, Kaback & Cohn, 1983a; Kanner & Metzger, 1983; Sagi-Eisenberg & Pecht, 1983). Neutrophils exposed to secretagogues exhibit a triphasic membrane potential response which involves external Na^+ (Korchak & Weissman, 1979; 1982). Transient elevations of intracellular ionized calcium are clearly seen with secretory stimuli (Stickle, Daniele & Holian, 1984) but these transients may simply be epiphenomena associated with some other effect of secretagogues. It is not at all clear calcium is acting in a true regulatory capacity (Stickle et al., 1984; McMillan, Macintyre, Beesley & Gordon, 1980; Goldstein, Hoffstein & Weissman, 1975).

The calcium hypothesis of stimulus-secretion coupling (Douglas, 1968) postulates that calcium entry through voltage-gated channels is required for exocytosis. Other ionic conductances therefore have a secondary effect on secretion by modifying the cell's electrical activity (Dubinsky & Oxford, 1984). Recent evidence indicates that, as in the case of the macrophage, this hypothesis cannot be generalized to include all cells. For instance, exocytosis can be dissociated from the voltage-dependent calcium current by a variety of methods including their differential sensitivities to low temperature (Atwater, Gonclaves, Malaisse, Rojas & Scott, 1984) and trifluoperazine (Clapham & Neher, 1984). Thus, calcium entry through voltage-dependent channels is not always absolutely necessary or sufficent for secretion. Nonetheless, most types of secretory cells show rapid electrical responses to secretagogues (Williams, 1981), suggesting that ionic conductances are somehow involved in secretion.

Recent studies with microelectrodes have linked voltage-dependent conductances to lymphocyte function albeit indirectly. In T lymphocytes, agents which block a voltage-dependent K^+ conductance also block mitogenic activation (Chandy, DeCoursey, Cahalan, Mclaughlin, & Gupta, 1984); the same conductance has been proposed to participate in the lethal reaction of cytotoxic T lymphocytes (Fukushima, Hagiwara & Henkart, 1984). In clonal B lymphocytes, voltage-dependent Ca^{2+} current increases in parallel with the cell's development of immunoglobin secretory capacity (Fukushima, Hagiwara & Saxton, 1984).

In summary, macrophages exhibit several activities that are in turn associated with changes in membrane potential. These electrical changes reflect intracellular ion dynamics that are believed to be involved in regulation of the mechanisms used to perform the cell's activities. Identifying and characterizing the ionic conductances of the macrophage's membrane are necessary steps in delineating the role of electrical activity in the macrophage's normal function and pathophysiology. Therefore, the ionic conductances of the macrophage's plasma membrane are the subject of my thesis research.

THE J774 CELL LINE

Any population of macrophages collected from a host contains a distribution of activation states which can complicate the analysis of macrophage physiology. Consequently, macrophage-like cell lines have been increasingly used as models of macrophages for physiological and biochemical studies (Ralph, 1980). Cell lines are tumors which can be grown *in vitro* using well characterized tissue culture techniques to produce large, homogeneous populations. One of the most attractive

features of cell lines as models of macrophage physiology is that genetic variants can be created and sublines with specific functional deficits selected for study (reviewed by Bloom et al., 1978).

Ralph et al. (1975 a&b) produced the J774 tumor cell line by adapting an induced mouse tumor for tissue culture through repeated passage back into mice. It has been extensively characterized and is currently one of the most widely used models of the macrophage. Table 1 lists properties of macrophages and describes their expression by J774 cells.

The spectrum of activities expressed by J774 cells most closely resemble those of a peritoneal macrophage elicited with a stimulus such as thioglycollate. This pattern of activities is intermediate between those of a resting and of an activated macrophage. The major physiolological distinction between J774 cells and primary macrophages (i. e. those obtained from normal animals) is that they don't express major histocompatibility antigens and as such cannot participate in Ia-restricted immune processes.

The J774 cell line has been used to investigate many areas of cell physiology. These include: a) receptor subclasses, b) the mechanism of Fc-receptor mediated phagocytosis, and c) the mechanism of the generation of reactive oxygen metabolites.

Immunoglobin molecules are composed of two domains; one is variable and specific for a given antigen while the other domain, the Fc-portion is constant and specific for class and sub-class of immunoglobin molecule. Macrophages have a receptor for this constant region called the Fc-receptor through which they internalize antigen/antibody complexes. It was assumed that each class and sub-class

TABLE 1

MACROPHAGE-LIKE FUNCTIONS OF THE J774.1 CELL LINE

Function	Activity	Reference
Phagocytosis		
Latex Beads	+	Ralph & Nakoinz, 1977
Zymosan	+	Ralph & Nakoinz, 1977
F_c Receptor-mediated	++	Snyderman et al., 1977
Secretion		
Plasminogen Activator	+	Bloom et al., 1978
Lysosyme	+	Ralph et al., 1977
Adenosine Deaminase	+	Snyderman et al., 1977
B-Glucuronidase	+	Snyderman et al., 1977
CSF (Interleukin 2)	+	Bloom et al., 1978
Aryl sulfatase	+	Bloom et al., 1978
Chemotaxis		
C5a	+	Snyderman et al., 1977
Respiratory Burst		
H2O2	+	Johnson et al., 1978
Superoxide	+	Greenberger et al., 1978
Adherence	++	Ralph et al., 1975
Enzymes		
Esterase	+	Greenberger et al., 1978
Cholesterol Esterase	+	Khoo, et al., 1981
Acid Phosphatase	+	Snyderman et al., 1977
O Alkaline Phosphatase	-	Greenberger et al., 1978
O Myeloperoxidase	-	Greenberger et al., 1978
Antibody-dependent cytotoxicity		
nonrestricted	+	Ralph et al., 1977
Ia-restricted	-	Ralph, 1980
Induction of T killer cells	+	Okada et al., 1978
Stimulation of IgG Production	+	Kishimoto et al., 1978

of IgG molecules had its own, unique, Fc-receptor which preserved specificity. The phagocytosis of monomeric IgG2a can be abolished by pretreatment of macrophages with trypsisn while preserving the ability to phagocytose IgG-coated erythrocytes (Unkeless & Eisen, 1975; Walker, 1977). This observation can be explained by two classes of receptors with different sensitivities to trypsin that are expressed simultaneously by the macrophage. The hypothesis was validated by using J774 cells which express IgG1, IgG3 and both IgG2a & IgG2b receptors (Schenk, Rosen, Diamond & Bloom, 1981). J774 cells express a particulary high density of Fc-receptors, over 500,000 per cell (Steinman, Mellman, Muller & Cohn, 1983). Cells were mutagenized and variant sublines were isolated that identified two classes of receptors; a trypsin-sensitive receptor which binds monomers of IgG2a and a trypsin-resistant receptor which binds aggregated IgG2b (Unkeless, Kaplan, Plutner & Cohn, 1979). Subsequent experiments using J774 cells have shown that phagocytosis via the two receptor types has different temperature sensitivities (Diamond, Bloom & Scharff, 1978).

Recent studies have shown that the IgG2b receptor acts as an ionophore. Upon crosslinking Fc-receptors, a Na^+-dependent depolarization was observed (Young et al., 1983a). In related studies, the Fc-receptor was isolated and inserted into planar lipid bilayers. Addition of immunoglobulin to the system resulted in discrete current fluctautions inidcating that the receptor-ligand complex formed ionic channels in the lipid bilayer (Young, Unkeless, Young, Mauro, & Cohn, 1983). Immunoglobin binding to J774 cells has been shown to evoke a transient rise in intracellular ionized calcium (Young, Ko & Cohn, 1984) using Quin 2, a flourescent indicator of ionized calcium. The calcium

presumably interacts with an actin/myosin-like system responsible for internalization of the receptor-ligand complex.

Investigations of receptor-mediated phagocytosis in J774 cells have suggested roles for several second messenger systems in addition to calcium. For instance, some J774 variants defective in Fc-receptor mediated phagocytosis can be restored to the wild type by addition of cyclic AMP (Muschel, Rosen & Bloom, 1977). Other studies suggest a role for calmodulin. Triflouroperazine (TFP), an inhibitor (in vitro) of calmodulin function, inhibits Fc-receptor mediated phagocytosis (Horwitz, Chia, Harracksingh, Orlow, Pifko-Hirst, Schneck, Sorbara, Speaker, Wilk & Rosen, 1981). TFP-resistant subclones of J774 cells obtained after mutagenesis are deficient in a calmodulin binding protein present in TFP-sensitive J774 cells (Speaker, Orlow, Sturgill & Rosen, 1983). Although the function of this protein is obscure at this point, the observation that its concentration is greater in activated peritoneal macrophages than in resident macrophages suggests a physiological role (Speaker et al., 1983). It is possible that receptor-mediated phagocytosis might be regulated by reciprocal effects of the calcium and cyclic nucleotide second messenger systems (Rassmussen & Goodman, 1977).

Highly reactive metabolites of oxygen are thought to be involved in the cytocidal mechanisms of many phagocytic cells (Klebanoff, 1982). The respiratory burst which produces these products can be elicited by a variety of cell surface stimuli, in particular, phorbol esters or aggregated immunoglobin. The metabolic requirements of the respiratory burst elicited by PMA have been studied both in wild type J774 cells and in a variant which does not produce either superoxide or peroxide (Damiani, Kiyotaki, Soeller, Sasada, Peisach & Bloom, 1980). It was

suggested that the defect in these cells is associated with a variant cytochrome b involved in the generation of superoxide anion (Kiyotaki, Peisach & Bloom, 1984). The fact that the parental cell line is resistant to the intracellular parasite Trypanosoma cruzi while the variant is sensitive indicates that these variant sub-lines may prove useful in the study of certain disease states.

MACROPHAGE ELECTROPHYSIOLOGY

Until the development of patch electrodes (discussed in the next section) the only method for directly recording membrane potential was the use of intracellular microelectrodes. Macrophages are particularly sensitive to electrode penetration damage, perhaps because of their relatively small size and large content of hydrolytic enzymes. Thus, recordings were difficult at best. The question of how much damage results from the penetration of the cell membrane with an electrode is difficult to assess. Consequently, progress to date in the field of macrophage electrophysiology has been comparatively slow.

The effect of penetration damage is apparent in the broad distributions of resting membrane potential (RMP) that have been reported for macrophages. Average RMP's, as determined with intracellular electrodes, have been reported from -13 mV to -72 mV (Gallin, Wiederhold, Lipsky & Rosenthal, 1975; Gallin & Livengood, 1980). The majority of observations lie between -26 mV to -40 mV. Gallin & Livengood (1980) observed a distinct bimodal distribution of RMP's in thioglycollate-elicited peritoneal macrophages; one group (approximately 66%) averaged -25 mV and the other group averaged -70 mV. The depolarized cells had linear current/voltage relationships whereas

hyperpolarized cells exhibited complex current/voltage relationships. They proposed that the linear, depolarized subpopulation might have been damaged cells while the hyperpolarized group represented healthy macrophages. An alternative explanation is that the two groups of macrophages were in different states of activation. Ince et al. (1983) analysed the electrical potential transient recorded upon microelectrode impalement of a wide variety of macrophages. Their general conclusion was that the true RMP of macrophages is considerably more negative than the sustained values recorded by intracellular electrodes. In conclusion, although it is generally assumed that depolarized RMP's indicate some degree of penetration damage, the contribution of other factors (state of activation, organ of origin, species, etc.) to the distribution of RMP's and ionic conductances remains unknown (Gallin and Sheehy, 1985).

Two voltage-dependent ion conductances have been studied with intracellular electrodes in macrophages (Gallin, 1984b). The first observed and best characterized is a conductance which produces a slow, transient hyperpolarization (Gallin, Widerhold, Lipsky and Rosenthal, 1975). These can occur spontaneously or can be elicited by mechanical, electrical or chemical stimuli. In contrast to slow hyperpolarizations (SH's) which are principally observed in depolarized macrophages, hyperpolarized cells often exhibit inward rectification (Gallin and Livengood, 1980). The resistance of an inward rectifying cell decreases for hyperpolarizing voltage steps (inward current) compared to depolarizing voltage steps (outward current). Thus, the ionic conductance which produces inward rectification increases with hyperpolarization.

Slow Hyperpolarizations

Slow hyperpolarizations are mediated by a well characterized K conductance that is sensitive to both voltage and intracellular ionized calcium. Gallin et al. (1975) first described the phenomenom in macrophages with the demonstration that the calcium ionophore A23187 produced hyperpolarizations which were inhibited by the calcium chelating agent EGTA. Slow hyperpolarizations are dependent on external potassium, and the effect is reduced by Verapamil, a Ca-channel blocker, (Olivera-Castro & Dos Reis 1981). Similarly, injection of Ca into a macrophage can evoke hyperpolarizations (Persechini, Araujo & Olivera-Castro, 1981). Slow hyperpolarizations are Cl-independent (Persechini et al., 1981) and can be blocked by external TEA (Dos Reis & Olivera-Castro, 1977). The calcium-dependent K conductance in the macrophage appears to be qualitatively similar to the calcium-dependent K conductance studied in neural tissues (Meech, 1978).

At present, the physiological role of the calcium-dependent K conductance in the macrophage is not understood. Calcium is known to mediate many activities in other cells and mechanisms have been proposed which link SH's to particular cell functions; however, experimental data to date is inconsistent with any of the proposed mechanisms. Phorbol myristate acetate (a secretagogue which works via protein kinase C which in turn is associated with elevated intracellular calcium), does not evoke SH's (Gallin, unpublished observations). Fc-receptor mediated phagocytosis of IgG-coated red blood cells does not produce SH's (Gallin, unpublished observations) though the mechanism is associated with elevated intracellular calcium (Young et al., 1984). On the other hand, Verapamil-inhibitable, repetitive hyperpolarizations are produced in

L-strain mouse fibroblasts during pinocytosis of high-density lipoproteins and phagocytosis of latex beads (Tsuchiya, Okada, Yano, Murai, Miyahara & Tanaka, 1981; Okada, Tsuchiya, Yada, Yano & Yawo, 1981). These responses are specific; they cannot be elicited by very-low-density lipoprotein or in non-phagocytic cells.

The availible evidence suggests that SH's reflect calcium entry induced by electrode damage to the macrophage membrane rather than Ca^{2+} dynamics associated with on ongoing physiologic process. This conclusion is supported by an analysis of the membrane potential transients recorded following microelectrode impalement. These studies indicated that SH's are not characteristic of unperturbed cells (Ince, Leijh, Meijer, van Bavel & Ypey, 1984). Thus, the hyperpolarizations are probably not spontaneous but are in fact evoked by calcium entering from outside the cell through a leak pathway induced by the electrode.

Inward Potassium Rectification

Rectifying conductances facilitate current flow in specific voltage ranges and are commonly associated with excitable cells. As previously mentioned, Gallin and Livengood (1980) observed that hyperpolarized mouse thioglycollate-elicited macrophages often exhibit complex steady-state current/voltage (I/V) relationships. I/V curves obtained under current clamp were defined by a high resistance transitional region bordered by two high conductance regions (inward and outward rectification) which produced an overall S-shape. These cells expressed inward rectification since membrane resistance was decreased by hyperpolarizing (inward) current. The transitional region of the I/V curve was insensitive to tetrodotoxin, cobalt, 4-aminopyridine and tetraethyammonium chloride but was sensitive to barium or rubidium.

An analysis which dissected the total inward rectifying current into its capacitative and ionic components demonstrated that the high resistance region recorded under current clamp indicated a negative slope region of the cell's I/V relationship (Gallin & Livengood, 1981). Membrane potential in such negative slope regions is inherently unstable under current clamp and therefore, a region of high resistance is created which separates the two regions of stable membrane potential. The negative slope region of the I/V curve was directly demonstrated later in mouse spleen macrophages by voltage clamp (Gallin, 1981).

The high resistance region in macrophages observed by Gallin & Livengood (1981) could have been produced by the inactivation of a potassium current or the activation of a sodium or calcium current. The effect of external barium, which eliminated the high resistance region and decreased the inward current, suggested that a voltage-dependent inward potassium current was involved. Egg and muscle cells have similar I/V relationships caused by by the activation of a barium-sensitive inward potassium current (Hagiwara and Takahashi, 1974; Standen and Stanfield, 1980). In these cells, the conductance has a unique relationship to external potassium; its activation shifts to depolarized potentials as $[K]_o$ is raised (Hagiwara & Takahashi, 1974; Leech & Stanfield, 1981). Demonstration of the same effect in macrophages (Gallin & Livengood, 1981) was strong evidence that inward rectification in macrophages was produced by a voltage-dependent inward potassium current similar to that described in egg and muscle cells. This current will be described in more detail in a later section of the introduction.

Despite considerable investigation, the inward rectifier has not been associated with any specific function in the macrophage.

Nonetheless, several important consequences of its expression are recognized (Gallin, 1984b). 1) Since the inward rectifier increases potassium permeability, its activation hyperpolarizes cells and therefore sets membrane potential at more negative values. This can have a substantial effect on other voltage-dependent conductances since RMP modulates a variety of membrane parameters including steady-state inactivation and the rate of activation of other voltage-dependent conductances (Hille, 1984). 2) Because of the high resistance region created as the inward rectifier turns off, voltage responses to depolarizing stimuli are greatly amplified relative to those produced by hyperpolarizing stimuli. Thus, depolarizing current will produce large voltage changes. In fact, cells with negative slope resistance regions can generate spike-like responses to injected current and may exhibit two stable states of RMP (Gallin & Livengood, 1980). In principle, the inward rectifier can produce switch-like control over other voltage-dependent processes. 3) The unique relationship between the inward rectifier and $[K]_o$ is particularly interesting in the context of the macrophage. Macrophages can act as scavengers of dead and dying tissue and are thus often found in sites of locally elevated [K]o. If the inward rectifier is associated with a particular macrophage function, it is likely that enhanced inward rectification may in turn enhance that function.

THE PATCH CLAMP TECHNIQUE

Erwin Neher has recently developed a new approach to membrane electrophysiology called patch clamping which is particularly suited to the study of small cells (Hamill, Marty, Neher, Sakmann & Sigworth,

1981). With this technique, the electrode is not pushed through the membrane (as in intracellular recording) but rather is held against the cell while gentle suction is applied. Under appropriate conditions and by a process that is not entirely understood, the membrane and electrode form a seal that is electrically (>10^9 ohm) and mechanically stable. Because the patch of membrane under the electrode is electrically isolated by the high resistance of the seal, small fluctuations of current flow can be recorded which represent the opening and closing of single ion channels (Hamill et al., 1981). This conformation is called the "cell-attached" mode. Alternatively, the electrode can be pulled away form the cell but the patch of membrane under the electrode remains attached (the "excised patch" mode). Single channel openings and closings can be resolved in this mode as well. A final option is to increase suction pressure while in the "cell-attached" mode to rupture the patch and acheive continuity between the inside of the cell and the inside of the electrode. This is referred to as the "whole-cell" recording mode. It is comparable to standard intracellular recording with the notable exception that the contents of patch electrode exchange with and eventually replace the cell's normal cytoplasmic contents. Some consequnces of this effect are addressed in a later section of the Discussion.

Many cell types, including macrophages, tolerate patch clamp procedures far better than penetration by an intracellular electrode (Marty and Neher, 1983). Consequently, patch clamping has become the approach of choice for the study of small cells such as leukocytes. Because it is a new technique, only a few reports on leukocytes have been published to date and most of these have dealt with lymphocytes.

Patch Clamp Studies on Leukocytes

The excised patch mode of recording has recently been used to directly demonstrate single, ion-selective channels in the macrophage. Gallin's study (1984a) on human macrophages determined the calcium and voltage dependencies of a potassium channel which probably mediates slow hyperpolarizations. The ability to control the compositions of the solutions bathing both sides of an excised patch of membrane makes this technique ideal for studying the sensitivity of a current to ionized calcium. As ionized calcium was increased, the voltage dependence of the potassium channel shifted to less depolarized voltages. Conclusions from these data can be extended by studies using patch electrodes in the cell-attached mode to determine the voltage-dependence of the calcium-activated potassium channel in situ. Since the channel's voltage dependence is a function of internal ionized calcium, channel activity is an indirect and non-invasive index of internal ionized calcium.

Schwarze and Kolb (1984) have used excised patches to demonstrate a large conductance, calcium-independent, chloride selective channel in mouse peritoneal macrophages. Analysis of the channel's opening and closing kinetics showed complex and strikingly steep voltage dependent rate constants.

Patch clamp techniques have confirmed observations of inward rectification in mouse spleen macrophages previously made with intracellular elctrodes but the conductance was not fully characterized in the study (Gallin, 1984b). A preliminary report has described inward rectification in a rat basophillic leukemia (Ikeda, 1984).

A voltage-activated outward potassium conductance which appears in mouse peritoneal macrophages cultured in vitro for at least 12 hours

was described by Ypey and Clapham (1984) using both single channel and whole-cell techniques. This current is also expressed in mouse and human lymphocytes (Fukushima & Hagiwara, 1983; DeCoursey, Chandy, Gupta & Cahalan, 1984; Matteson & Deutsch, 1984). While the outward current has yet to be associated with a macrophage function, numerous studies in lymphocytes have linked it to mitogenic activation (Matteson & Deutsch, 1984; DeCoursey et al., 1984) and killing mechanisms (Fukushima, Hagiwara & Henkart, 1984). Extrapolation of the channel's calcium permeability from that of other cell types indicates that this channel could account for the calcium transients associated with lymphocyte mitogenesis (Cahalan, Chandy, DeCoursey & Gupta, 1985). This is crucial since no voltage-dependent calcium channels have been observed in T lymphocytes. The properties of the outward rectifier in macrophages seem to be similar to those in lymphocytes (Ypey & Clapham, 1984; Cahalan et al., 1985) but the inward rectifier in macrophages has not been studied in as great a detail.

In contrast to macrophages and T lymphocytes, B lymphocyte mouse myeloma cell lines exhibit a calcium current (Fukushima & Hagiwara, 1983; Fukushima et al., 1984). A non-secretory myeloma line exhibited a small calcium current (Fukushima & Hagiwara, 1983) while secretory myeloma lines exhibited a prominent voltage-dependent calcium current (Fukushima et al., 1984). The time course of development of the voltage-dependent calcium current in secretory cells paralleled that of immunoglobin secretory activity. This suggests that the calcium current is associated with the imunoglobulin secretory mechanism.

VOLTAGE-DEPENDENT POTASSIUM CURRENTS

My thesis describes two voltage-dependent potassium currents in the J774.1 macrophage-like cell line. These currents, one inward and one outward, are similar to currents observed in primary macrophages (Gallin & Livengood, 1981; Ypey & Clapham, 1984) and in other cell types. The subsequent section details the characteristics of these ionic conductances in other cells so that they may be compared to those of J774.1 cells.

INWARD RECTIFICATION

Potassium currents which activate with hyperpolarization are widely distributed and have been described in skeletal and cardiac muscle, neurons, macrophages and oocytes. This current was first studied by Katz (1949) in frog skeletal muscle and was first called anomalous rectification because it was in the opposite direction to the delayed rectification described at the same time by Hodgkin, Huxley & Katz (1952). It is now well documented that the current changes as a function of time, voltage and external K.

Time Dependence

Activation of the inward rectifier shows both instantaneous and time-dependent components; the relative contribution of the two varies considerably among cell types. Activation is slowest in the oocyte of the starfish Mediaster (Hagiwara, Miyazaki & Rosenthal, 1976) where the time constants of activation are an order of magnitude or more greater than those observed in tunicate eggs or frog skeletal muscle (Ohmori, 1978; Leech & Stanfield, 1981; DeCoursey, Dempster & Hutter, 1984).

The rate of activation is voltage-dependent and increases with hyperpolarization. Actual time constants have only been determined in frog skeletal muscle. Experiments performed at low temperature ($5^{o}C$) yielded time constants on the order of 15 to 20 msec for a 20 mV hyperpolarizing step (Hestrin, 1981; Leech & Stanfield, 1981) while a value of 3 msec was obtained at room temperature (DeCoursey, Dempster & Hutter, 1984). The fastest activation rates appear to be in ventricular heart muscle where activation is essentially instantaneous (less than 1.5 msec; Sakmann & Trube, 1984). The inward current rectifies for both its instantaneous and time-dependent components (Hestrin, 1981).

Voltage Dependence

The voltage-dependence of the inward conductance (G) follows a Boltzman function of membrane potential (V_m) and the potassium equilibrium potential (E_k) as first proposed by Hodgkin and Horowicz (1959):

$$G/G_{max} = 1/[1+\exp((V-V_h)/v)] \qquad V = V_m-E_k \qquad (1)$$

where V_h is a constant which locates the conductance curve along the voltage axis, v is a constant which determines the slope of the relationship and G_{max} is the limiting value of the inward rectifying conductance. This description applies to egg cells (Hagiwara & Takahashi, 1974) and skeletal muscle (Hestrin, 1981).

Inactivation

The decrease in the inward potassium current with time was first investigated by Adrian, Chandler & Hodgkin (1970) in frog skeletal muscle and most subsequent studies have used this preparation. In skeletal muscle, much of the decline can be attributed to a decrease in the potassium electrochemical gradient as intracellular potassium

redistributes into the transverse tublular system during long hyperpolarizing steps. The validity of this mechanism is supported by experiments which demonstrate that the decline in inward current with time is reduced by raising $[K]_o$ (Almers, 1972 a&b). As $[K]_o$ is increased, the effect of redistributing of internal potassium is proportionally reduced and the potassium gradient is thereby less changed. Other mechanisms are also evident. Larger hyperpolarizations elicit another, faster component to the time-dependent decrease which has a much greater sensitivity to temperature. Replacement of external Na with choline in either skeletal muscle or tunicate eggs eliminates the second component of inactivation (Ohmori, 1978; Standen & Stanfield, 1979). Recent single channel studies in ventricular muscle have demonstrated inactivation without changes in the K electrochemical gradient and in the absence of external Na (Sakmann & Trube, 1984). In summary three general mechanisms are believed to contribute to the decline of inward rectifying current with time: a) collapse of the driving force due to K redistribution, b) open channel block by weakly permeant ions and c) a third channel state (in addition to open and closed) corresponding to true inactivation.

Ionic Dependence

The ionic composition of the media has several striking effects on the properties of the the inward rectifier. As already noted, its activation shifts to more positive potentials as $[K]_o$ is raised. The voltage-dependence of its activation was initially described as being relative to the K driving force (Hagiwara & Takahashi, 1974). With the development of internal perfusion techniques it has been shown that activation actually depends on $[K]_o$. Little to no changes are observed

as $[K]_i$ is varied, (Hagiwara & Yoshii, 1979; Hestrin, 1981; Leech & Stanfield, 1981). Thus K_o has a direct effect on channel gating. In addition to K_o-dependent gating, peak conductance of the inward rectifier is proportional to the square root of $[K]_o$ in starfish eggs (Hagiwara & Takahashi, 1974). This property has been confirmed in other systems and is intrinsic to the single channel (Sakmann & Trube, 1984; Fukushima, 1982).

Intracellular perfusion studies have shown that internal sodium is required for the activation of the inward potassium current (Hagiwara and Yoshii, 1979) although the channel has no measurable permeabilty to sodium (Hagiwara and Takahashi, 1974). Increasing internal sodium increases the inward rectifying current. The relationship conforms to Michaelis/Menton kinetics, implying the presence of a sodium binding site (Hagiwara & Yoshii, 1979).

OUTWARD RECTIFICATION

The delayed rectifier studied by Hodgkin and Huxley (1952 a,b,c & d) in the squid axon was the first voltage-dependent potassium current to be characterized using voltage clamp techniques. Following a step depolarization, an outward potassium current activates a few milliseconds after the sodium current (hence the name "delayed"). The rate and extent of activation of the current increase with depolarization. Since that classic series of papers, delayed rectification has been extensively studied in skeletal muscle (Adrian, Hodgkin & Chandler, 1970), and nerve (Goldman & Schauf, 1973; Thompson, 1977).

The general mathematical formalism describes the time course of the delayed potassium conductance, G(V,T), as the product of the maximum

conductance (G_{max}) times two dimensionless variables; an activation variable n, raised to the fourth power and a first order inactivation variable, k:

$$G(V,T) = (G_{max})x(n^4)x(k) \qquad (2)$$

where G(V,T) is the potassium conductance as function of time and voltage. The activation and inactivation variables are both voltage- and time-dependent.

Variability

It should be stressed that there is considerable variation among delayed-type rectifiers from different cell types. The prototype delayed rectifier of the squid axon inactivates only for extremely long depolarizations and then only partially (Ehrenstein & Gilbert, 1970) but that of mollusc nerve cell bodies (Aldrich, Getting & Thompson, 1979; Connor & Stevens, 1971), coelenterates (Hagiwara, Yoshida & Yoshii, 1981) and skeletal muscle (Duval & Leoty, 1978; Adrian, Hodgkin & Chandler, 1970) inactivates within a few seconds although to a varying extent. The exponent of the activation variable (n) ranges from 1 (frog skeletal muscle: Argibay, Hutter and Slack, 1973) through 2 (mollusc nerve cell body: Thompson, 1977; worm axon: Goldman & Schauf, 1973) to 4 (Squid axon: Hodgkin & Huxley (1952d); rat skeletal muscle: Beam & Donaldson, 1983). The significance of these variations is unclear, particularly in the case of the different activation exponents since kinetic descriptions are very sensitive to experimental design. In any event, there are few generalizations regarding delayed rectifiers that will hold to all cell types. A further consideration is that other currents often appear in the same voltage ranges which complicate the kinetic analysis of the delayed rectifier (Connor & Stevens, 1971).

Time Dependence of Activation

The delayed rectifier activates faster with increasing membrane depolarization in all tissues where it has been studied. For a step to the same membrane potential, the time to half-peak of the outward current can vary by over an order of magnitude. For a step to 0 mV, human lymphocytes reach half-peak in 2.5 msec (Cahalan et al., 1985), mouse macrophages at 10 msec (Ypey and Clapham, 1984) and mollusc neural somata at 50 msec (Connor and Stevens, 1971). This variation is the product both of variation between species and variation between experimental conditions. For steps to 0 mV rat skeletal muscle outward current reaches half-peak in 1 msec at 37°C but takes 30 msec at 7oC (Beam and Donaldson, 1983).

Voltage Dependence of Activation

Activation of the delayed rectifier begins at voltages positive to -60 mV in frog skeletal muscle (Argibay, Hutter & Slack, 1973), -40 in rat skeletal muscle (Beam & Donaldson, 1983; Pappone, 1980), -20 in mollusc cell bodies (Connor & Stevens, 1971) and -10 in coelenterates (Hagiwara, Yoshida & Yoshii, 1981). The extent of activation increases sigmoidally with voltage. Due to contamination by other currents it has not been routinely possible to determine where activation plateaus. In the few systems where the delayed rectifier can be studied in isolation (Hodgkin and Huxley, 1952d; Cahalan et al. 1985) the voltage dependence of the conductance (G) has been shown to follow a Boltzman function of membrane potential (V_m):

$$G/G_{max} = 1/[1 + \exp((V_m - V_h)/v)]^4 \qquad (3)$$

where V_h is a constant which locates the conductance along the voltage axis, v is a constant which determines the slope of the relationship and

G_{max} is the limiting value of the outward conductance. The fourth power dependencies of the time and voltage dependencies of the conductance have generally been interpreted as implying that channel's gating process entails the concerted action of four particles. Cole and Moore (1960) found, after large conditioning hyperpolarizations, that the outward (potassium) current in squid axon was best fit by n^{25} kinetics rather than the conventional n^4 description. This finding has cast doubt on the interpretation that n^4 kinetics suggest 4 gating particles.

Inactivation

Analysis of inactivation of the delayed rectifier as a first order process often reveals two exponential decay processes. The best studied example is in the mollusc nerve cell body (Aldrich, Getting & Thompson, 1979). They found that the faster component of decay (τ=570 msec) accounted for about 90% of inactivation while another, slower (τ= 3.5 sec) process accounted for the rest. Inactivation is complete only in frog skeletal muscle (Adrian, Chandler & Hodgkin, 1970), whereas in most other cell types, the steady state current is about 30% of the peak.

Compared to the kinetics of onset of inactivation recovery from inactivation proceeds very slowly (τ=30 sec). Thus it is critical for an experimenter to allow sufficient time between pulses in order to ensure complete recovery. This phenomenom, "cumulative inactivation", has been suggested to provide a significant modulating influence on the shape of action potentials produced during repetitive firing.

The delayed rectifier inactivates at potentials negative to those at which it activates. In contrast to the inward rectifier, the potentials at which the outward rectifier inactivates are near the RMP in some tissues. This phenomenom is called steady-state inactivation. As

with most other aspects of this conductance, the contribution of steady-state inactivation varies with cell type. In human lymphocytes the conductance inactivated by 50% at -70 mV (Cahalan et al., 1985) while in mouse macrophages inactivation is absent at voltages negative to -60 mV (Ypey & Clapham, 1984).

Ionic Dependence

Delayed rectifiers are largely unaffected by the ionic composition of the internal and external bathing solutions. The channel is slightly permeable to calcium in the squid axon (Inoue, 1981) and has been proposed as route for the entry of calcium during lymphocyte activation (Cahalan et al., 1985). Flouride, used as the intracellular anion in some patch clamp studies, has been reported to alter gating properties of the channel (Adams and Oxford, 1983).

PURPOSE OF STUDY

The purpose of this study is to validate the J774.1 cell line as a model for investigations of the role of voltage-dependent ion conductances in macrophage function. The electrophysiological properties of the J774.1 cell line were determined both in long-term adherent culture and as a function of time after the cells were transfered from suspension to adherent culture. The properties of J774.1 cells were also compared to previously determined properties of primary macrophage cultures.

MATERIALS & METHODS

CELL CULTURE

J774.1 cells obtained from the American Type Culture Collection (Rockville, MD) were maintained in tissue culture medium containing RPMI 1640 supplemented with 10 units/ml penicillin, 10 uG/ml streptomycin (Difco Labs, Detroit, MI), 0.03% glutamine (Sigma, St. Louis, MO), and 10% fetal bovine serum (Hyclone, Logan UT). RPMI 1640, a synthetic culture medium originally designed for growing leukemic cells, was developed at Roswell Park Memorial Institute, Buffalo, NY.

Stock cultures were maintained as a non-adherent population in spinner flasks treated with Sigmacote (Sigma, ST. Louis, MO). Cultures were kept at 37 ^{o}C in an incubator containing 7% CO_2 and 93% air. Media was changed every 2 to 3 days. Cells were 12-14 microns in diameter. Prior to experiments, cells were withdrawn and plated in 35 mm tissue culture petri dishes (Corning Glass Works, Corning, NY). To avoid the possibility of recording from electrically coupled cells, confluent cultures were not studied.

Genetic stability of the cell line was judged by comparison of present cultures to cells that were frozen and maintained in liquid nitrogen. There was no evidence of genetic drift in the culture. A recent assay of the cell line performed by the National Cancer Institute (Fort Detrick, Frederick, MD) determined that the cells were free of Mycoplasma virus.

IRRADIATION

For studies of irradiated cells, the AFRRI cobalt-60 radiation source was used. Cells, suspended in Teflon jars, were bilaterally exposed to 20 gray gamma radiation at 5 gray/minute and were plated immediately thereafter.

ELECTRICAL RECORDINGS

Intracellular electrode studies

Cells were viewed through a Zeiss inverted phase contrast microscope. Cells were maintained at 35-37^{o}C by an electrically heated block mounted in the microscope stage; a thin layer of mineral oil atop the recording media prevented evaporation of the media. Intracellular recordings were obtained in a balanced salt solution containing (in mM) 145 NaCl, 4.6 KCl, 1.6 $CaCl_2$, 0.6 $MgCl_2$, 10 N-2-hydroxyethypiperazine-N'-ethanesulfonic acid (HEPES, buffered to pH 7.3), 10 glucose and 5% bovine serum albumin (Fraction V, Sigma, St Louis, MO).

Intracellular recordings of membrane potential were obtained by a WPI model M-707 amplifier (WPI, New Haven, CT) with a 10^{11} ohm input impedence. Current injected by the amplifier flowed through two princal resistive elements, the electrode and the cell's membrane; the total voltage response is the sum of the IxR drops across each element. Since the resistance of intracellular electrodes can be on the order of the cell's membrane resistance, signifigant distortion of the cell's voltage response can occur if the electrode's reponse cannot be eliminated. The amplifier used for these studies automatically subtracts the IxR drop across the electrode from the total voltage response of the circuit

through the use of an active bridge curcuit. The electrode's resistance is determined in the bath before cell penetration by adjusting the bridge circuit's resistive element until the voltage output is flat for injected current, at this point the bridge is said to be balanced.

Fiber-filled K-Acetate or KCl (3M) electrodes (resistance 60-180 Mohms) were pulled on a Brown-Flaming model P-77 micropipette puller (Sutter Inst. Co., San Francisco, CA) and connected to the amplifier through WPI Ag/AgCl half cells. Membrane resistance was monitored by periodically passing small hyperpolarizing current pulses (<.2 namp). Current was measured by a virtual ground current meter (Connor and Stevens, 1970) connected to the bath by a 0.3M KCl/Agar bridge and half cell. Current and voltage were simultaneously displayed on an oscilloscope for photography and recorded on a strip-chart recorder (Gould Instruments, Lanham, MD). Some experiments were also recorded on magnetic tape (Vetter model B FM tape recorder, A. H. Vetter, Rebersburg, PA).

A recording was rejected if it failed to meet any of the following criteria: a) upon penetration of a cell, the voltage trace showed a sharp break from the baseline, b) current pulses administered by the recording electrode showed that the cell had a membrane resistance and capacitance clearly distinguishable from those of the electrode, c) RMP and membrane resistance were stable for at least 5 min, d) the voltage trace returned to within 5 mV of the baseline upon withdrawing the electrode from the cell, e) the bridge was still balanced after withdrawal, f) visual confirmation that the electrode was indeed inside the cell during the recording and g) that the cell maintained normal morphology throughout the recording (in particular, cells which formed

large vacuoles were rejected).

Patch clamp studies

Recordings were made using the whole cell variation of the patch electrode voltage-clamp technique (Hamill et al., 1981). Electrodes were filled with (in mM) 145 KCl, 10 NaCl, 1.0 $MgCl_2$, 1.1 EGTA, 0.1 $CaCl_2$ (pCa = 7.7) (pCa = 7.7) and 10 mM HEPES, and brought to pH 7.3 with KOH. The bath solution contained 155 NaCl, 4.6 KCl, 1.6 $CaCl_2$, 0.6 $MgCl_2$, and 10 HEPES (pH = 7.30) and was maintianed at rom temperature (22-25^{o}C). Ionic concentrations were used to calculate reversal potentials because at these ionic strengths, internal and external activities of Na and K were within 2% of each other (Fujimoto & Kubota, 1976). In experiments where external K was raised, KCl was substituted for NaCl.

Patch electrodes were fabricated from standard hematocrit capillary tubes using a two step process performed on a vertical pipette puller (Model 700C, David Kopf Instruments, Tujunga, CA). The first pull, performed at high heat, was 60 mm. The electrode was recentered in the heating coil and a lower heat was applied. Electrodes were fire polished and then coated with beeswax to reduce electrode capacitance; resistances ranged from 2 to 10 Mohm. No compensation for the resistance of the patch electrodes was performed. Since the maximum amplitude of currents measured in this study were <0.5 nAmps, the error due to voltage drop across the electrode was no more than 5 mV and usually much less. Seal resistances ranged from 5 to 20 x 10^9 ohm.

The output of a Dagan 8900 patch-clamp amplifier (Dagan Corp., Minneapolis, MN) was displayed on the oscilloscope and a strip chart recorder. An Ampex PR2200 FM tape recorder was used (Ampex Corp.,

Redwood City, CA). Data were filtered through a 1 Khz low pass filter included in the amplifier.

The circuits used in a patch clamp amplifier saturate when the input voltage is greater than 10 volts and may be non-linear at 5 volts. The input voltage is product of the current needed to clamp the membrane potential times the resistance of the amplifier's headstage resistor ($V = I \times R$). Since currents as high as 1×10^{-9} ampere were anticipated, a resistance as high as 10×10^{9} ohm could have been used; a feedback resistor of 1×10^{9} ohm was selected to ensure that the amplifier was always well within its linear operating range.

Resting membrane potential was estimated as that potential at which no current flowed across the cell membrane (the "zero-current potential"). Unless otherwise noted, cells were maintained at the initial zero-current potential and voltage steps were separated by 10 seconds.

DATA ANALYSIS

I/V relations

I/V relations of J774.1 cells were determined by injecting current or voltage steps or by injecting a voltage ramp. Under current clamp, I/V curves were calculated by measuring the voltage responses to constant current pulses of variable amplitude. A WPI Stimulus Isolator was used to generate pulses, pulse length and delay were controlled by a WPI Interval Generator. Current amplitude, set by the stimulus isolation unit, was independently measured by the virtual ground current meter. Under voltage clamp, current was measured in response to voltage steps. The magnitude of the step was controlled by a WPI Stimulus Isoltion Unit;

step length and delay were controlled by a WPI Interval Generator. Alternatively, I/V relationships were generated using slow ramps (dV/dT = 20mV/sec) and plotted directly onto an X-Y recorder (Hewlett Packard 7015B, Hewlett Packard, San Diego CA).

The standard convention of plotting the independent variable on the X-axis and dependent variable on the Y-axis has been followed for constructing I/V relations. Therefore, I/V relations of cells studied with intracellular electrodes (current clamp) have current as the X-axis whereas I/V relations of cells studied with patch electrodes (voltage clamp) have voltage as the X-axis.

Statisitics and Curve Fittings

All values presented are means +/- SEM (n = number of replicates).

Curve fitting was done on a VAX 750 computer (DEC, Boston, MA) using the RS1 software package (BBN Inc., Cambridge, MA). This package uses the Marquandt-Leverberg method (Bard, 1974) of least squares analysis of non-linear functions.

RESULTS

INTRACELLULAR ELECTRODE STUDIES

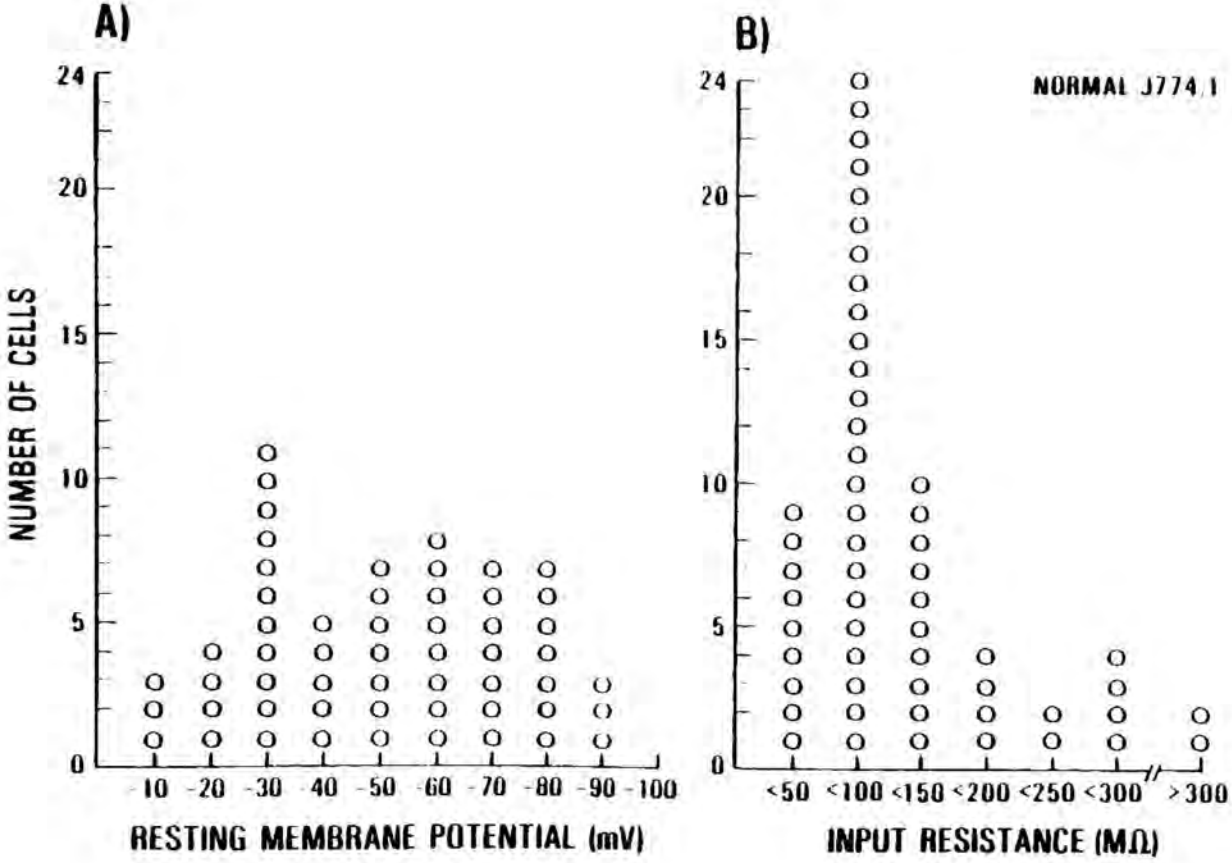
Resting Membrane Potentials

The resting membrane potentials (RMP's) of normal J774.1 cells exhibited a broad, unimodal distribution ranging from -8 to -91 mV (Fig. 1a) with a mean value of -50.8 mV +/-3 (n=55) as determined by intracellular microelectrode current clamp. The most depolarized cells undoubtedly included some damaged by penetration. Rather than arbitrarily excluding some recordings, all which satisfied the acceptance criteria detailed in the Materials & Methods have been included in Fig. 1. Cells with hyperpolarized RMP's were usually initially depolarized. Hyperpolarization generally occurred in a single discrete period; long periods of gradual hyperpolarization were exceptional. The time between penetration and stabilization of RMP was variable but usually did not exceed 5-10 min. A RMP stable for 5 minutes after this stabiliization period was included. There was no apparent correlation of RMP to age after plating.

Resistances & Time Constants

As calculated for small (<0.2 nAmp) hyperpolarizing current pulses administered from RMP, membrane resistance (R_m) also showed a broad distribution of values (Fig. 1b) which ranged from 25 to 600 Mohm with a mean value of 117 +/- 13 (n = 55). No systematic interrelation was found for RMP & R_m, high & low R_m's were observed at all RMP's. This

Figure 1. Passive electrophysiological characteristics of 56 J774.1 cells recorded in HEPES-buffered balanced saline solution. Data were obtained from 51 different culture dishes, cells plated 4 to 14 days prior to recordings. A.) The distribution of resting membrane potential. B.) The distribution of membrane resistance.


A)
B)
NORMAL J774.1
NUMBER OF CELLS
0
5
10
15
20
24
-10 -20 -30 -40 -50 -60 -70 -80 -90 -100
RESTING MEMBRANE POTENTIAL (mV)
<50 <100 <150 <200 <250 <300 >300
INPUT RESISTANCE (MΩ)

observation suggests that the depolarized cells are not entirely artifacts of membrane damage (since these cells would therefore all have low values of R_m). The average time constant (= $R_m C_m$, eqn. 5b of Appendix I) was 19.7 +/- 2.3 msec (n = 35). A specific membrane resistance of 19 +/- 3.1 Kohm-cm^2 was calculated from the values of and R_m observed for each cell using eqn. 8 of Appendix I.

Rectification

Current/voltage relationships for five cells recorded from the same dish are shown in Fig. 2. The slope of the I/V curve (R_m = V/I), is independent of voltage in three of the cells. In 2 cells, R_m decreased for voltages beyond -70 mV (inward rectification). Two cells have relatively depolarized values of RMP, two have relatively hyperpolarized values and one is intermediate. Note that no single cell can be taken as representative of all the rest. This suggests that the differences in electrophysiological properties of J774.1 cells are not the result of dish to dish variation in culture conditions.

An index of a membrane's voltage-dependent conductances is the non-linearity of its I/V relationship. The non-linearity of the I/V relations of J774.1 cells studied with intracellular electrodes was examined by comparing R_m calculated between -100 mV to -80 mV with that calculated from -60 mV to -40 mV. For this study, a cell was scored as rectifying if R_m between - 60 to -40 mV increased by greater than a factor of 1.5. This analysis was performed on the cells of Fig. 1 and is presented in Fig. 3. Essentially, this index scores I/V curves for the presence of a transitional region. For example, both of the cells in Fig. 2 with a RMP of -70 have ratios greater than 1.5 whereas the rest

Figure 2. Current/Voltage relationships of five J774.1 cells recorded in the same dish, each symbol represents a different cell. Voltage shift measured after charging of membrane capacitance is plotted on the ordinate and the amplitude of the injected current pulse is plotted on the abscissa.

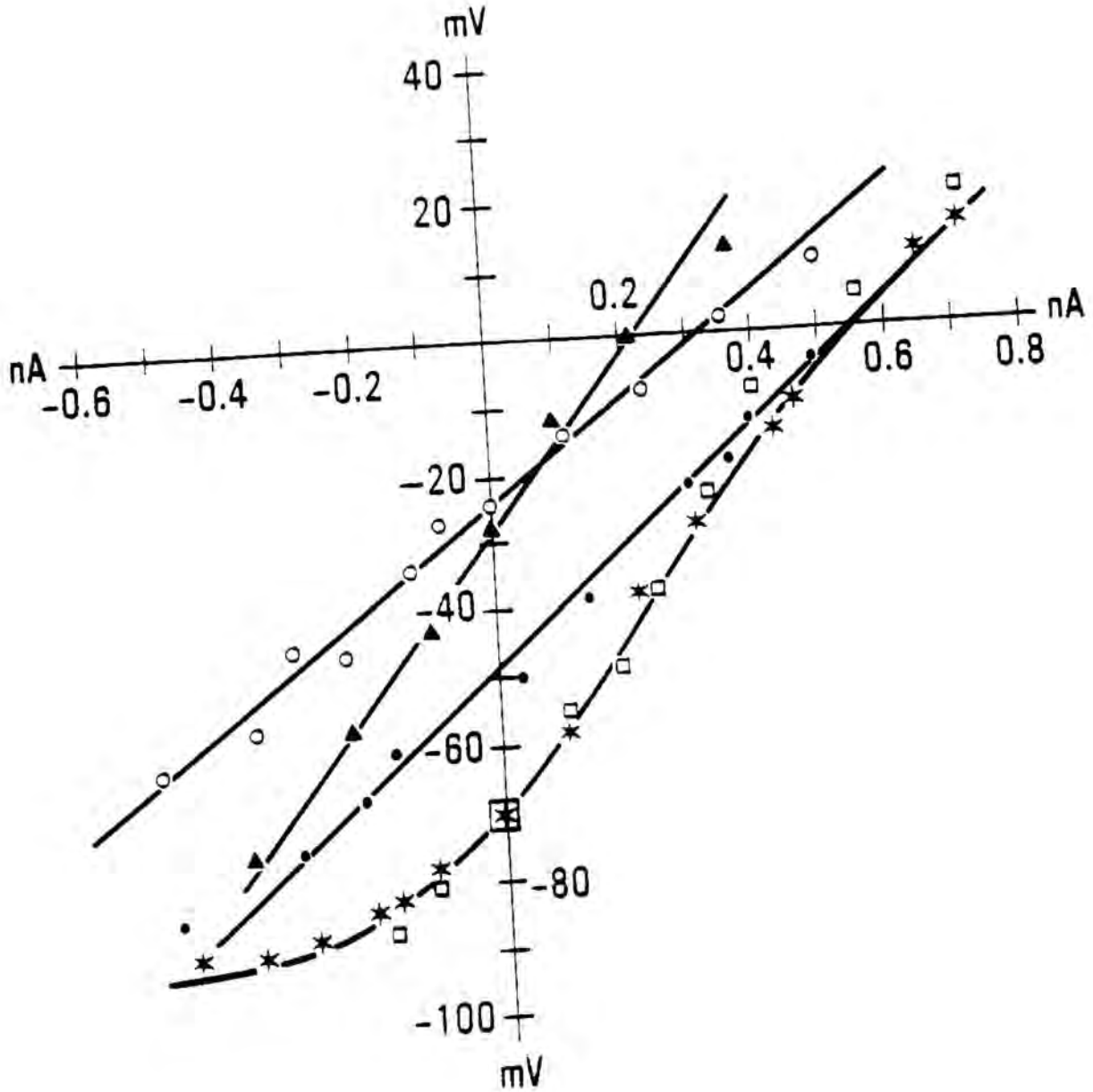
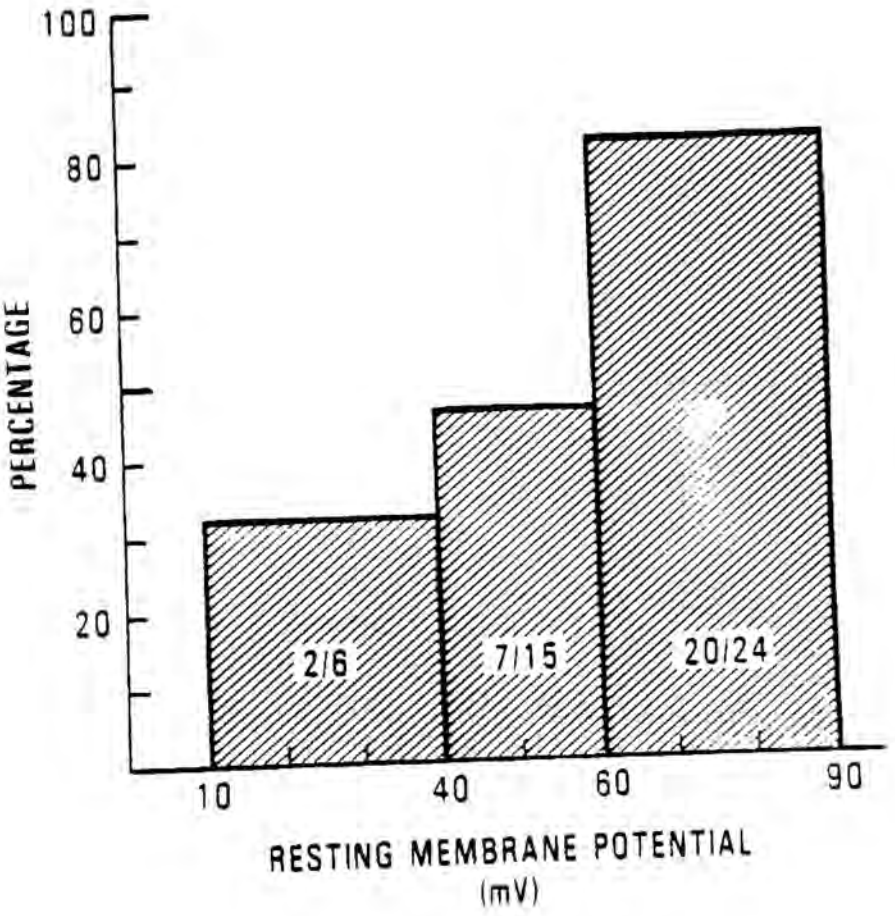

mV
40
20
-20
-40
-60
-80
-100
mV
nA
-0.6
-0.4
-0.2
0.2
0.4
0.6
0.8
nA

Figure 3. Per cent J774.1 cells with a high resistance region plotted as a function of RMP. High resistance is defined as membrane resistance calculated between -40 and -60 mV greater than 1.5x that between -80 and -100 mV. Each bar includes the number of cells meeting this criterion over the number observed.


100
80
60
40
20
PERCENTAGE
2/6
7/15
20/24
10
40
60
90
RESTING MEMBRANE POTENTIAL
(mV)

score negative. The two hyperpolarized cells in Fig. 2 reflect the general pattern shown in Fig. 3 in that J774.1 cells which express inward rectification are those with more negative RMPs. This result was noted earlier by Gallin & Livengood (1980) in thioglycollate-induced peritoneal macrophages.

The effect of $BaCl_2$ on a cell exhibiting both inward and outward rectification is shown in Fig. 4. In 4.6 mM $[K]_o$, the I/V curve is S-shaped, with a transitional zone between -60 and -40 mV. Increasing $[K]_o$ to 11.6 mM shifted the activation of the inward rectifying current to more positive potentials; outward rectification (evident as an increase in slope) continued to be evident at voltages positive to -40 mV. Subsequent addition of $BaCl_2$ eliminated the transitional zone. Outward rectification was still apparent at voltages positive to 0 mV.

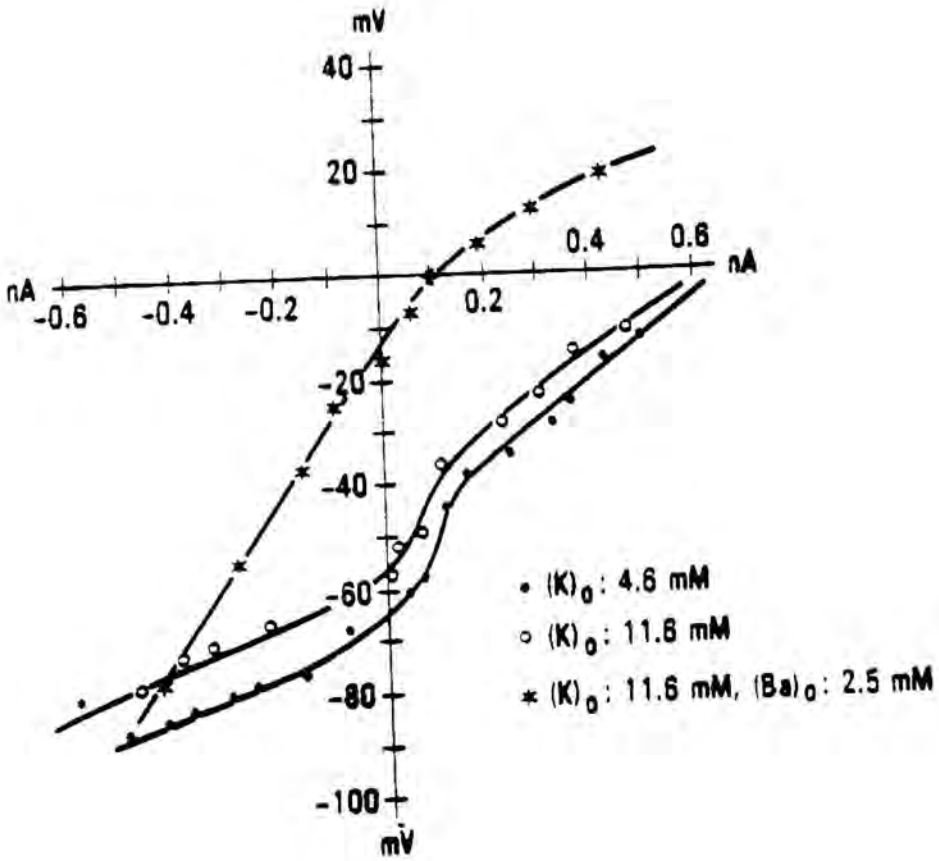
$BaCl_2$ produced a depolarization in 8 of the 9 cells investigated. The average value of the shift was 27.6 +/- 6.94 mV.

Slow Hyperpolarizations

Transient hyperpolarizations were observed in some cells, most commonly immediately after penetration. Rythmically hyperpolarizing cells were rarely observed. Usually only a few cycles of hyperpolarization (totaling a minute or two) occurred just after penetration whereas hyperpolarizations which occurred later sometimes cycled for tens of minutes or greater.

Transient hyperpolarizations in J774.1 cells have features consistent with the Ca^{2+}-mediated K conductance described in primary macrophages (Gallin, 1984). 1) Current pulses applied during a hyperpolarizating cycle produced smaller voltage changes than those

Figure 4. Current/Voltage relationship of a J774.1 cell in 4.6 mM $[K]_o$, 11.6 mM K_o, and 11.6 mM K_o + 2.5 mM $BaCl_2$.


mV
40
20
-20
-40
-60
-80
-100
mV
nA
-0.6
-0.4
-0.2
0.2
0.4
0.6
nA
• $(K)_o$: 4.6 mM
○ $(K)_o$: 11.6 mM
* $(K)_o$: 11.6 mM, $(Ba)_o$: 2.5 mM

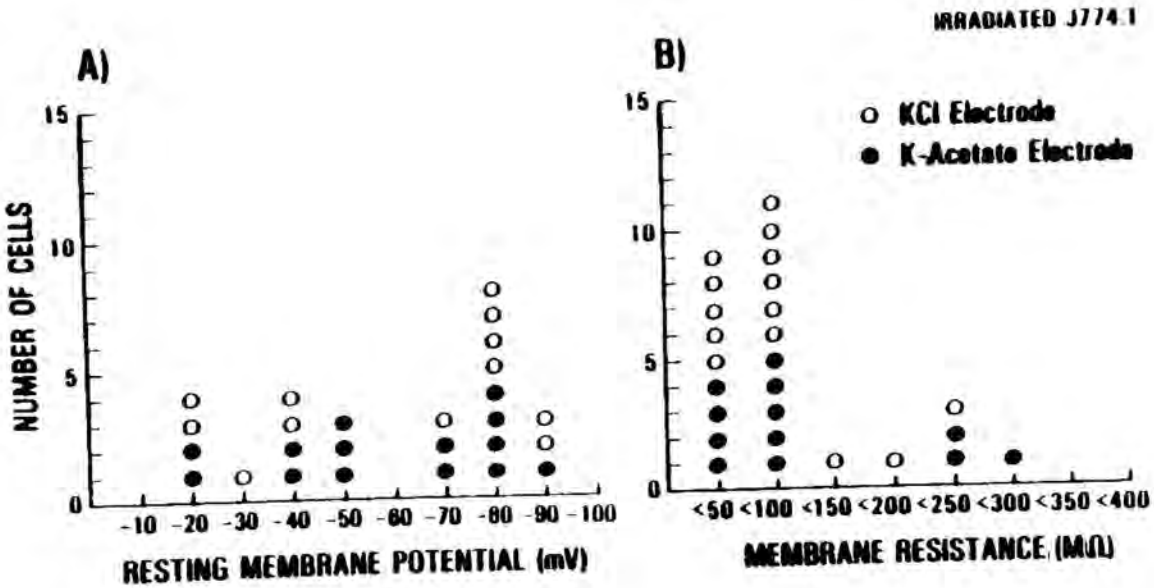
applied between hyperpolarizations, indicating increased membrane conductance. 2) The magnitude of the transient hyperpolarization was dependent on the RMP. As the RMP was depolarized, the magnitude of the transient hyperpolarization increased. These features suggest that transient hyperpolarizations in J774.1 cells are due to a transient conductance increase to potassium. Attempts to increase the amount of Ca^{2+} entering non-hyperpolarizing cells (by increasing bath Ca^{2+} or biasing RMP by injection of DC current) did not elicit transient hyperpolarizations.

Irradiated J774.1 Cells

Because of the small size of these cells, much of the scatter in the distribution of RMP & R_m was believed to have been the result of cell damage. It was therefore of interest to investigate techniques which might increase cell size. Recent work by Gallin et al. (1985) has shown that J774.1 cells approximately double in size 3 days after 20 gray of gamma radiation. This dose blocks cell division (as measured by incorporation of tritiated thymidine) but not cell growth.

The baseline electrophysiological characteristics of irradiated J774.1 cells (2000 Rad ^{60}Co, 500 Rad/min.) are presented in Fig. 5. While the distribution of RMPs of irradiated J774.1 cells is nearly as broad as that of normal cells, it does not contain as many of the very depolarized values. The distribution is seemingly bimodal; aproximately half the cells were more negative than -68 mV (14/26, average -80.7 +/- 1.77) with the others positive to -54 mV (12/26, average -36.2 +/- 3.5). The average RMP of the entire population was -60.4 mV +/- 4.7 and the average R_m was 94.5 +/- 15 Mohm. Neither of these values was observed to

Figure 5. Passive electrophysiological characteristics of 27 Gamma-Irradiated J774.1 cells (2,000 Rad Co^{60}, 500 Rad/min) recorded with microelectrodes filled with either 3M KCl or 3M K-Acetate. Each point was obtained in a different dish. A.) The distribution of resting membrane potential. B.) The distribution of membrane resistance.


IRRADIATED J774.1
A)
B)
NUMBER OF CELLS
15
10
5
0
-10 -20 -30 -40 -50 -60 -70 -80 -90 -100
RESTING MEMBRANE POTENTIAL (mV)
KCl Electrode
K-Acetate Electrode
<50 <100 <150 <200 <250 <300 <350 <400
MEMBRANE RESISTANCE (MΩ)

depend on age post-irradiation up to 2 weeks. The specific resistance calculated using eqn. 8 of Appendix I was 27 +/- 5.8 Kohm-cm^2 (n= 15).

Blatt & Slayman (1983) found that filling microelectrodes with potassium salts that diffuse less rapidly than KCl significantly improved the quality of electrical recordings obtained from a small cell. They proposed that this was due to reduced salt leakage from the microelectrode into the cell. The filled circles in Fig. 5 a&b show data obtained in irradiated J774.1 cells after substitution of 3M K-Acetate for the conventional 3M KCl microelectrode filling solution. This did not effect a qualitative change in the distribution of either RMP or R_m.

Calculations using eqn. 7 of Appendix I (= 26 msec) showed that the surface area of irradiated J774.1 cells almost doubled within 4 days (1.73-fold +/- 0.33, $p<0.05$). The fact that fewer depolarized cells were observed is probably due to the greater resistance of larger cells to damage by electrode penetration.

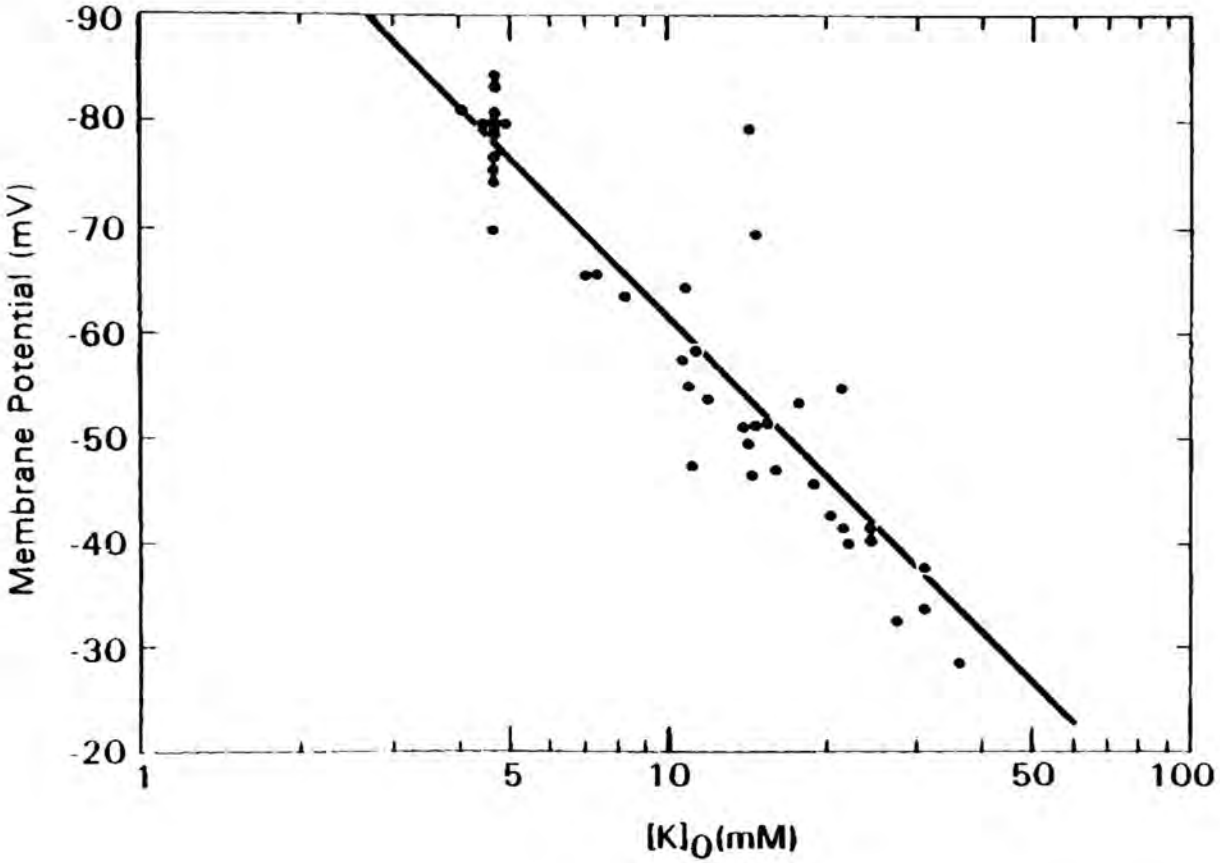
PATCH CLAMP STUDIES

Resting Membrane Potential

Initial studies using patch electrodes were performed on long-term adherent cultures (>24 hrs) of J774.1 cells because they were judged more comparable to previous intracellular microelectrode studies of macrophages (Gallin, 1981; Gallin & Livengood, 1981). The potential at which no current flowed across the cell membrane (zero-current potential) was used as an estimate of RMP. The zero-current potential of 37 cells averaged -77.6 mV +/- 0.78 (SEM); values ranged from -66 to -85 mV. The observation that the zero-current potential was within 10% of the potassium equilibrium potential (E_k = -85 mV, eqn. 2 of Appendix II assuming RT/F = -56, $[K]_o$ = 4.6 and $[K]_i$ = 150 mM) indicates that shunt to ground across the pipette/cell seal or conductance to ions other than K^+ was no more than 10% of the whole cell conductance. Zero-current potentials of long-term adherent J774.1 cells determined within seconds of attaining the whole-cell conformation were routinely stable for greater than 30 minutes and as long as 90 minutes.

To determine the relationship between the RMP and the potassium equilibrium potential, studies were done in which the extracellular potassium was varied and the zero-current membrane potential was measured. The data obtained from 12 different cells each exposed to three or four sequentially increasing $[K]_o$ are shown in Fig. 6. The linear regression line drawn through these points has a slope of -49 +/- 0.51 mV/tenfold increase in $[K]_o$ (correlation coefficient -.90) which is signifigantly different from a slope of -56 mV/tenfold increase in $[K]_o$

Figure 6. Relationship between membrane potential (estimated as zero-current holding potential) and $[K]_o$ as determined with whole-cell patch clamp. Data were obtained from 12 different cells exposed to three or four sequential increases in $[K]_o$.

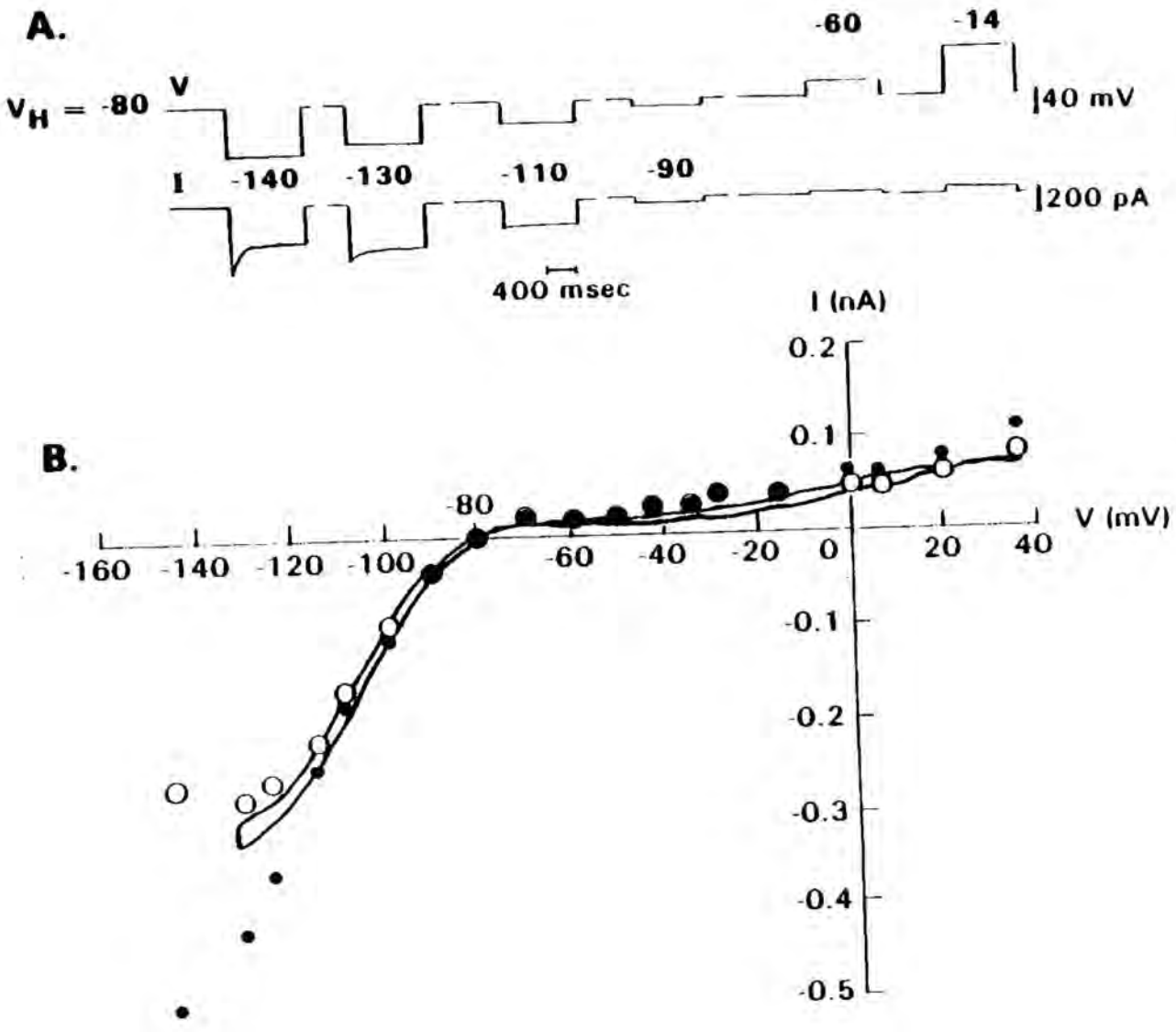

-90
-80
-70
-60
-50
-40
-30
-20
Membrane Potential (mV)
1
5
10
50
100
[K]O(mM)

predicted by the Nernst equation for a potassium electrode ($p < .01$).

Current/voltage Relationships

All long-term adherent J774.1 cells exhibited marked inward rectification. Figure 7a depicts the current responses of a cell to voltage steps taken from its zero-current potential of -80 mV. Hyperpolarizing voltage steps produced large inward currents which activated rapidly. At membrane potentials more negative than -120 mV, the currents decreased with time. The current elicited by depolarizing steps was considerably smaller and exhibited no time dependence. The I/V relationship of this cell obtained from both voltage steps (circles) and a voltage ramp (solid line, dV/dT = 20 mV/sec) is shown in Fig. 7b. The apparent peak measured 10 msec after the beginning of the step (a time when the ionic current could be clearly distinguished from the capacitative transient) and the steady-state current obtained with voltage steps are both shown in Fig. 7b. Since current was not time-dependent in the voltage range of +40 to -110 mV, the peak and steady-state curves as well the ramp-derived curve are identical. However, at voltages negative to -110 mV they diverge. In other studies in which ramps to more negative potentials were applied, a prominent hysteresis was evident (see Fig. 8 c and d) due to time-dependent inactivation of the inward current. The cell analysed in Fig. 7 had a slope conductance (calculated using eqn. 1 of Appendix II) of 9.6 nS for inward current (calculated from -100 to -130 mV) and a slope conductance of 0.9 nS for outward current (calculated from 50 to -40 mV). The leak conductance of long-term adherent cells, calculated from -50 to -40 mV, was 1.01 nS +/- .12 (mean +/- SEM, n=22).

Figure 7. I/V characteristics of a long-term adherent (>24 hr) J774.1 cell. A) Current responses to a series of voltage steps from holding potential (V_h) = -80 mV. B) Current/voltage relationship of cell shown in Fig. 7a. Solid circles represent current measured 10 msec after start of step. Open circles represent current measured at end of step (1 sec). Voltage steps were applied every 10 sec. Solid line represents current generated by injection of a voltage ramp (dV/dT = 20mV/sec).

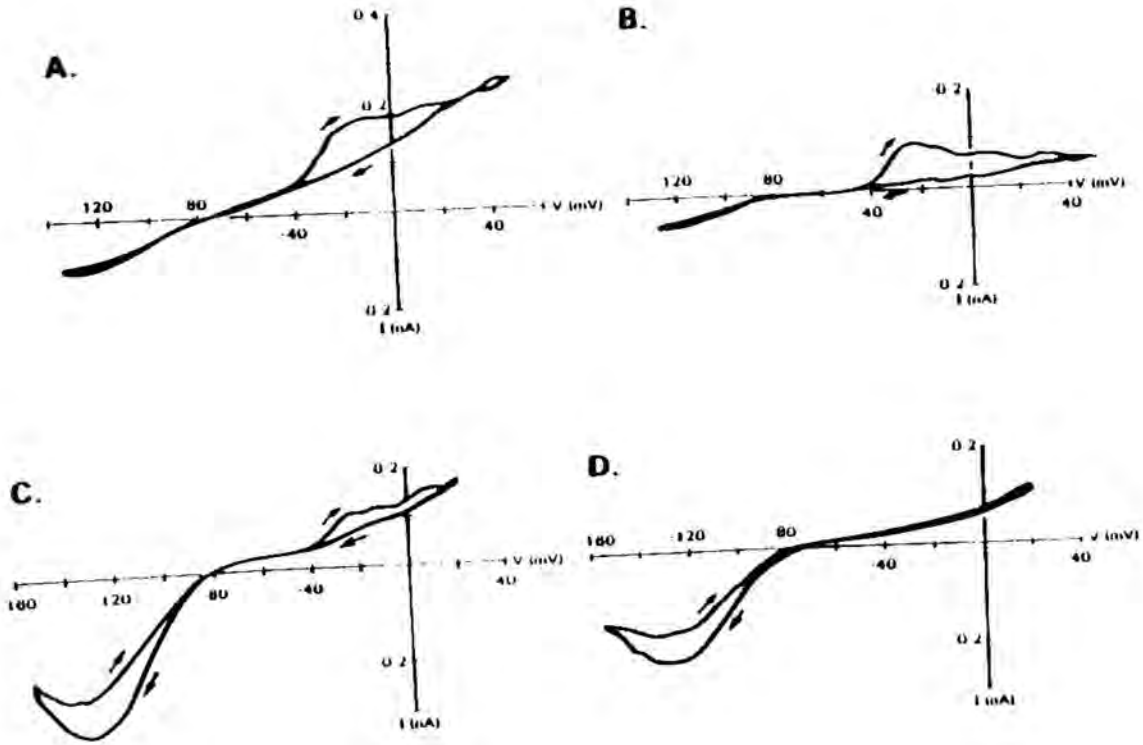

A.
-60
-14
V_H = -80
V
40 mV
I
-140
-130
-110
-90
200 pA
400 msec
B.
I (nA)
0.2
0.1
-80
V (mV)
-160
-140
-120
-100
-60
-40
-20
0
20
40
-0.1
-0.2
-0.3
-0.4
-0.5

Effect of Adherence on RMP and I/V Relations

The resting membrane potential of nonadherent J774.1 cells as measured with tetraphenylphosphonium ion, an indirect probe of membrane potential, has been reported to be significantly more positive than the values obtained in this study with adherent J774.1 cells (Young et al., 1984). To investigate the cause of this difference, J774.1 were grown in spinner culture and plated shortly before recordings were performed. The zero-current potentials of cells studied at 30 minutes to 24 hours after plating were indistinguishable from those of long-term adherent cultures (-66 mV or more negative), indicating that J774.1 cells probably hyperpolarize rapidly upon plating.

However, the cells' I/V curves varied considerably with time after plating. Four I/V curves representative of the pattern seen in the 195 cells analyzed by patch electrodes in this study are shown in Fig. 8. Cells showed no evidence of inward rectification immediately after plating (<1 hour) although small, inactivating, outward currents were sometimes observed. The increase in the slope of the I/V curve at voltages positive to -40 mV in the depolarizing limb of the ramp in Fig. 8a reflects the activation of an outward current. The returning limb of the ramp was flat due to inactivation of this outward current. This inactivating outward current was observed up to 8 hr after plating (Fig. 8 b and c) but was absent in cultures more than 10 hr old (Fig. 8d). The outward current is described in a later section of this paper. Inward rectification usually became evident about 2 to 4 hr after plating (Fig. 8c) and was the characteristic feature of I/V curves from cells plated 24 hr or longer (Fig. 8d). Experiments with voltage steps

Figure 8. Current/voltage relationships of J774.1 cells obtained by voltage ramp at various times after plating from suspension culture. Holding potential (V_h) was zero-current potential. A) 1 hour after plating. V_h = -77 mV. B) 3 hours. V_h = -78 mV. C) 7 hours. V_h = -81 mV. D) >24 hours. V_h = -72 mV.


A.
B.
C.
D.
120
80
-40
40
160
I (nA)

confirmed the results obtained with ramps.

While the inward rectifier was always present in long-term cultures, expression of the outward current was highly variable. The fraction of cells exhibiting the outward current 2 to 4 hr after plating varied from 90% to 20%. The consistent observation was that the inward current was not evident until 2 or more hours after plating. The inactivating outward current was seen between 1 and 10 hr post-plating and, with only one exception, never thereafter.

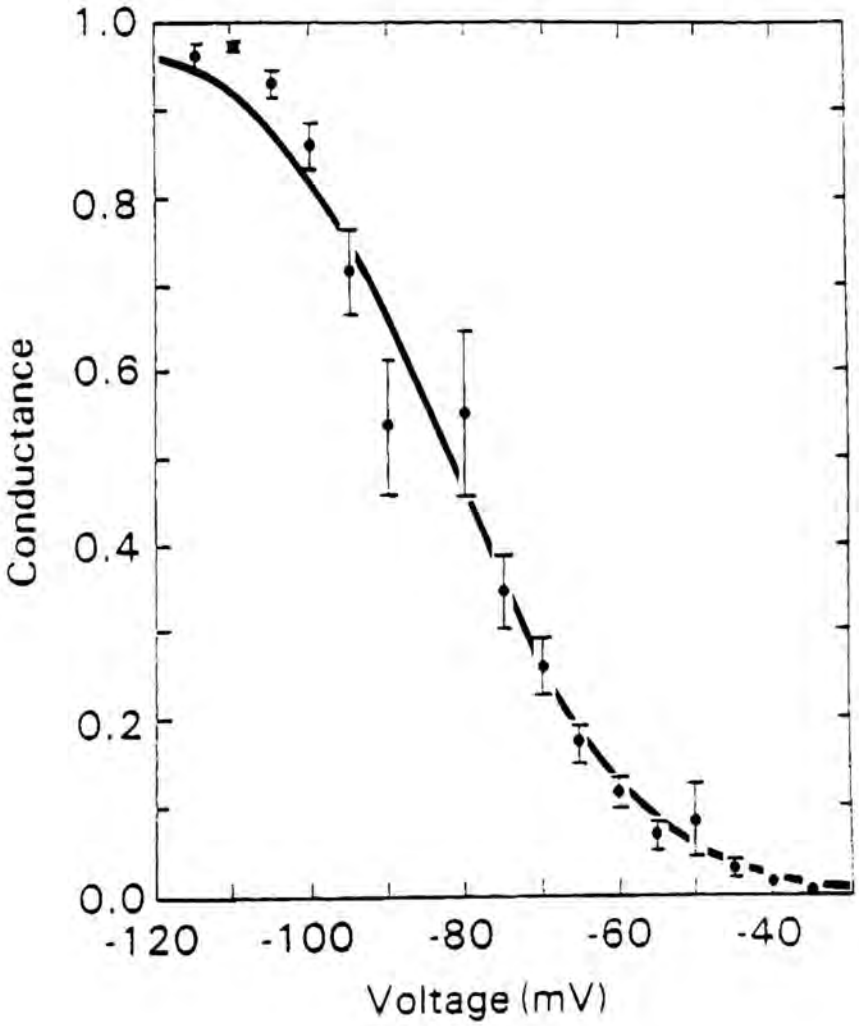
All analyses of the inward and outward currents (with the exception of Fig. 19) which are presented later in this paper were all performed on data from stabilized cells. Apart from an occaisional transient increase in leak current during the first 10 minutes of a recording, zero-current potentials were stable throughout an experiment.

Inward Rectification

The Conductance/Voltage Relationship

Analyses performed using I/V curves obtained with voltage steps demonstrated that ramp-derived curves provided valid peak instantaneous I/V relationships up to -110 mV (Fig. 7b). Therefore the chord conductance of the inward current (calculated using eqn. 4 of Appendix II) was calculated from I/V curves obtained from ramps. Leak conductance, determined at -30 mV, was subtracted. The reversal potential of the inward rectifying current was assumed to be the E_k (-85 mV). The conductance/voltage relations of 12 long-term adherent cells (each cell's maximum conductance normalized to 1.0) obtained in 4.6 mM K_o are presented in Fig. 9. The conductance was substantially activated at rest (about 39% at -77 mV) and thus contributed to the cell's resting

Figure 9. Chord conductance versus voltage of 12 long-term adherent J774.1 cells. Conductances were leak subtracted (calculated at -30 mV) and normalized by peak conductance for each cell. Reversal potential was assumed to be -85 mV. Each point is the mean +/- SEM. Solid line conforms to eqn 1.


1.0
0.8
0.6
0.4
0.2
0.0
Conductance
-120
-100
-80
-60
-40
Voltage (mV)

membrane potential. At potentials negative to -110 mV the conductance of inward rectifier appeared to decrease (see also Fig. 10b). This was an artifact resulting from inactivation of the conductance during the ramp because experiments with steps demonstrated that the inward conductance peaked by -110 mV and remained constant for more negative voltages. With the exception of the drop in conductance at voltages negative to -110 mV, the parameters determined from peak currents in response to voltage steps agreed closely with those determined from current responses to voltage ramps. This indicates that inactivation at voltages positive to -110 mV during the course of the voltage ramp was negligble.

The solid line drawn through the points in Fig. 9 represents an empirical relation reported by Hagiwara for the anomalous rectifier in egg cells (Hagiwara & Takahashi, 1974). This relationship is described by the equation:

$$G = 1/(1 + \exp(V-V_h)/v)) \quad V = V_m-E_k \qquad (1)$$

where v is a constant that characterizes the slope of the relationship and V_h represents a constant that locates the curve along the voltage axis; at $V = V_h$ the conductance is half-maximal. $V = (V_m-E_k)$ is the driving force of the current. The data shown in Fig. 9 were fit by nonlinear least squares regression. Values of 2.85 +/- .08 and 11.57 +/- .25 were obtained for V_h and v respectively, (coefficient of multiple determinations = 0.993). The relation is steep near rest, fractional activation drops from 66% at -90 mV to 26% at -70 mV. The average peak conductance of the cells in Fig. 9 was 6.57 +/- 1.06 nS. Conductance/voltage characteristics of long-term adherent cells were routinely stable for greater than 30 minutes.

Effects of K_o

The inward rectifying potassium conductance in egg and muscle cells is a function of both the membrane and the external potassium concentration (Hagiwara Takahashi, 1974; Leech & Stanfield, 1981). To determine if this was also the case for macrophages, the effect of varying external potassium was investigated in J774.1 cells. The I/V curves of a single cell obtained after equilibration in solutions containing four sequentially increasing $[K]_o$ are shown in Fig. 10a. In addition to depolarizing the cell, raising $[K]_o$ increased the slope conductance for inward currents and shifted the voltage dependence of inward rectification to the right. In Fig. 10b the cell's conductance/voltage relations are displayed for each of the four potassium concentrations shown in Fig. 10a. Each set of points was fit to equation 1; V_h and v determined at each $[K]_o$ were not substantially different from the values at $[K]_o$ = 4.6 mM when the appropriate values of RMP and peak conductance were substituted. A log-log plot of peak conductance versus potassium is shown in Fig. 10c; linear least squares regression gives a slope of 0.56 (r=.97). This series of experiments was performed on a total of eight cells, the average slope of the log $[K]_o$ versus log peak conductance relationship was 0.41 +/- 0.07. Thus, there is an approximate square root relationship between the peak inward rectifying conductance and $[K]_o$, which is similar to that observed in egg and muscle cells (Hagiwara & Takahashi 1974; Leech & Stanfield 1981).

Inactivation

The I/V relations in Fig. 7 a and b show that inward current was time independent for voltage steps up to -90 mV but decreased with time

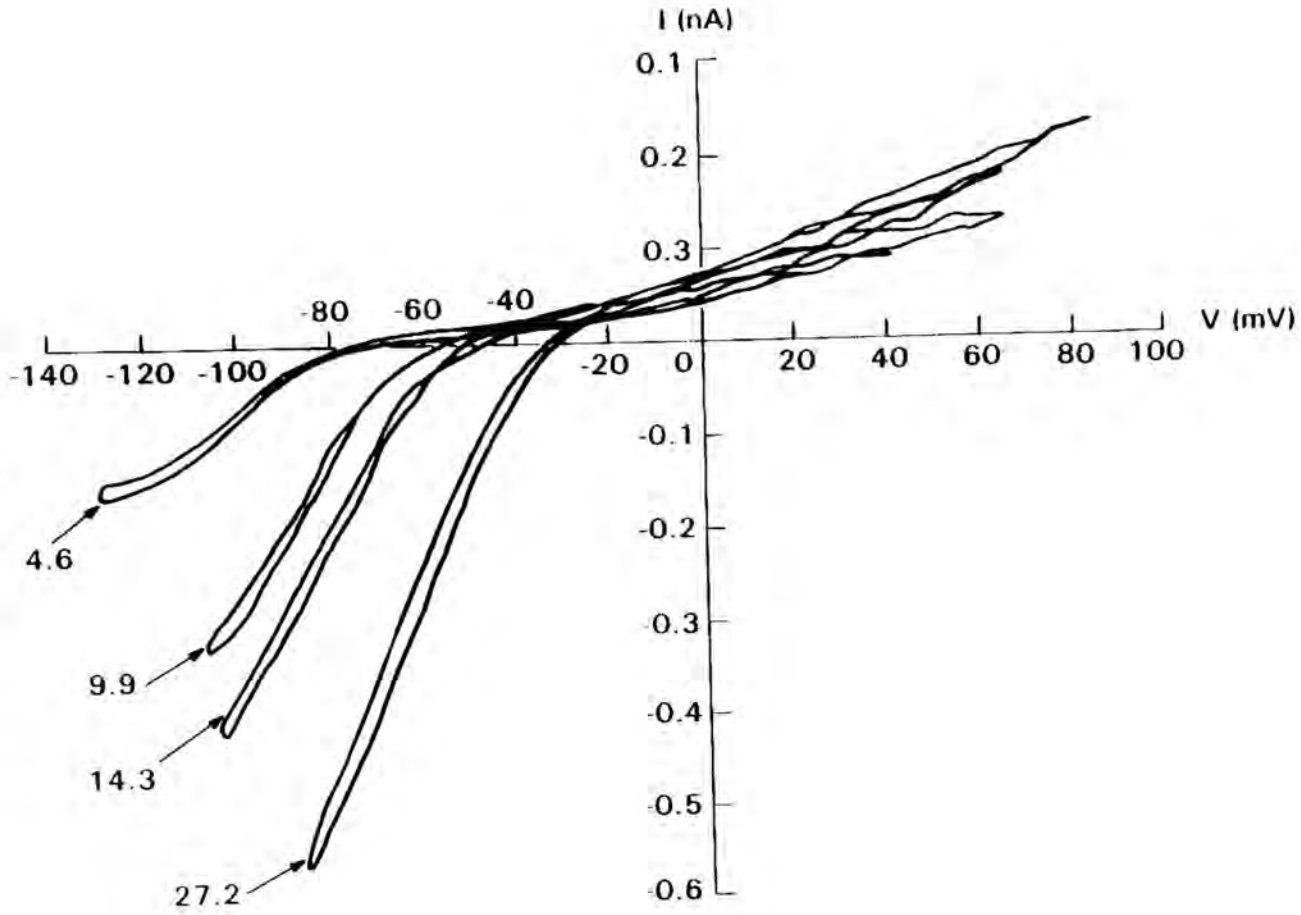
Figure 10. K_o dependence of inward rectification.

A) Current/voltage relationships of the same cell taken sequentially in 4.6, 9.9, 14.3 and 27.2 mM K_o. Holding potentials were zero-current potentials; V_h = -75, -56, -50 and -33 mV, respectively.

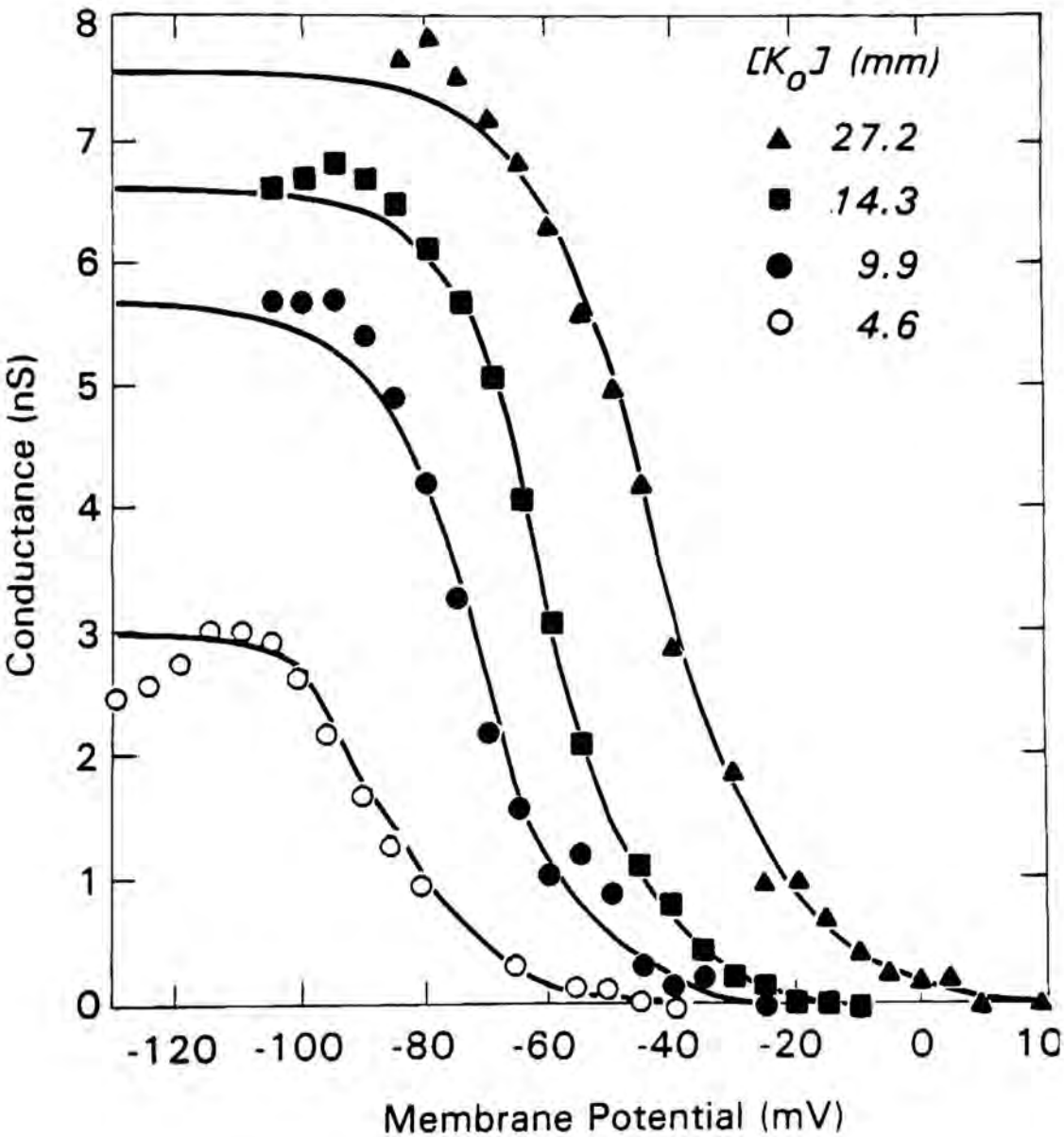
B) Chord conductance versus voltage for each $[K]_o$ calculated as in Fig. 9; conductance 40 mV positive to rest was used as a measure of leak.

C) Log peak conductance versus log K_o. Data in panels b and c were fit by least squares regression ($r>.97$).

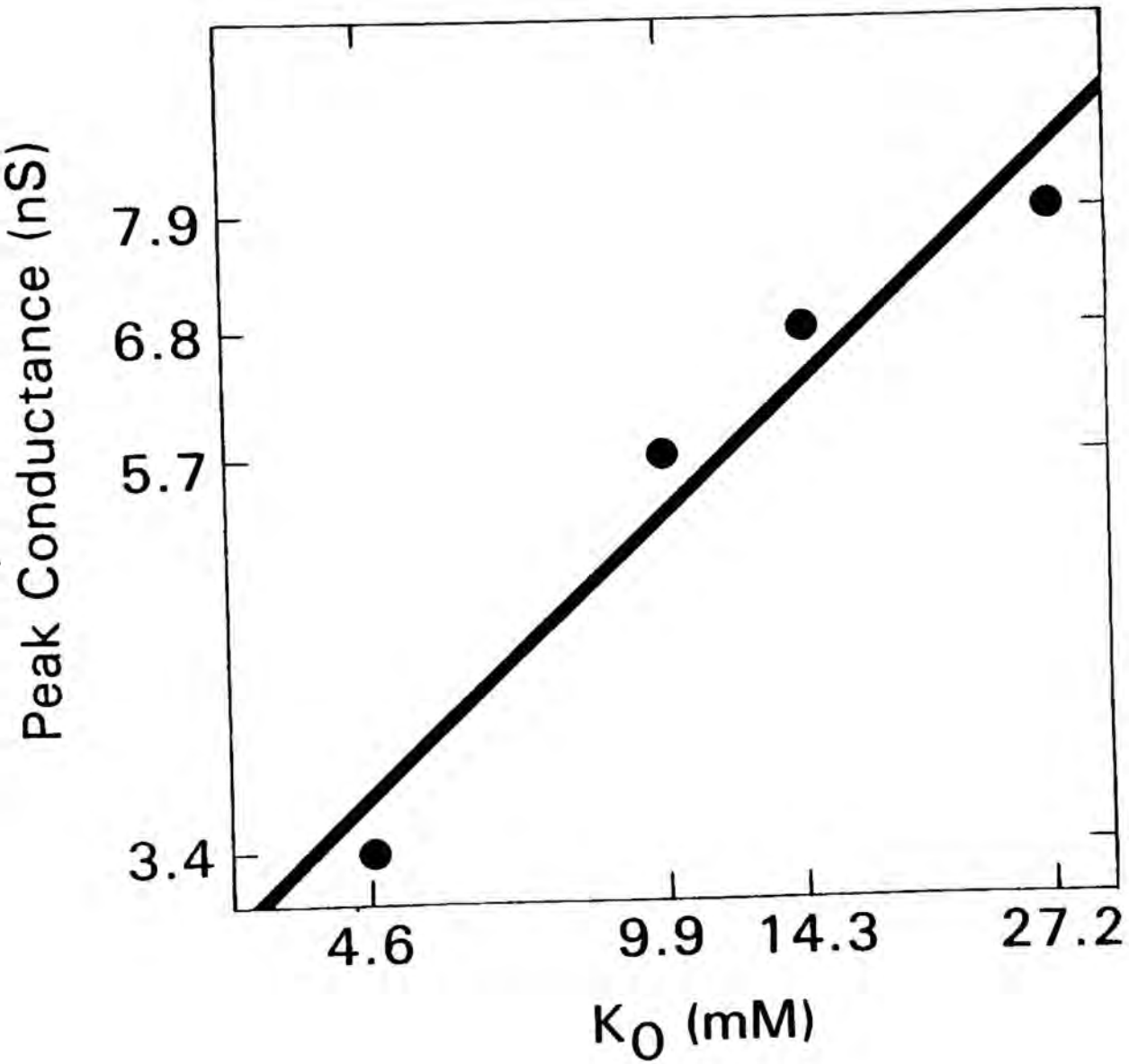
A


I (nA)
0.1
0.2
0.3
-80
-60
-40
V (mV)
-140
-120
-100
-20
0
20
40
60
80
100
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
4.6
9.9
14.3
27.2

B


[K$_o$] (mm)
27.2
14.3
9.9
4.6
Conductance (nS)
Membrane Potential (mV)
0
1
2
3
4
5
6
7
8
-120
-100
-80
-60
-40
-20
0
10

C



at more negative voltages and suggest that the inactivating component of total inward current increases with hyperpolarization. The ratio of the steady-state current (Iss) to the apparent peak current (Ip) is plotted versus voltage for eight cells in Fig. 11. The peak was measured 10 msec after initiation of the voltage step, at which time the capacitive transient had clearly ended. The data in Fig. 11 show that inactivation occured at voltages negative to -100 mV (note the decrease in current at the end of a pulse compared to the beginning) and that the inactivating component of inward current increased with hyperpolarization. At the most negative voltages studied (-150 to -175 mV), the steady-state inward current was still greater than leak. It was not possible to determine whether the ratio of I_{ss}/I_p plateaued at very negative voltages since cells rarely survived voltage steps to -150 mV or greater.

Fig. 12a shows the time-dependent current (where I_t is current as a function of time and I_{ss} is the steady-state current) measured from current records as a function of time for four different voltage steps. The current relaxations were fit by single exponentials ($p<.01$). The time constants determined for voltage steps to -154, -145, -136, and -113 mV were 58, 159, 202, and 317 msec respectively. Fig. 12b shows the time constants of decay as a function of voltage for eight different cells. Time constants decreased with increasing hyperpolarization.

Blockers

Addition of barium (2-3 mM) to the bathing medium blocked inward rectification in J774.1 cells which is consistent with its action on skeletal muscle and tunicate egg (Standen and Stanfield, 1978; Hagiwara and Takahashi, 1974). The I/V curves from a cell before and after the application of barium are shown in Fig 13a. The I/V curve following

Figure 11. Steady-state current/peak current (I_{ss}/I_p) versus voltage. Data from eight cells. Open circles represent values obtained from the cell shown in Fig. 12c.

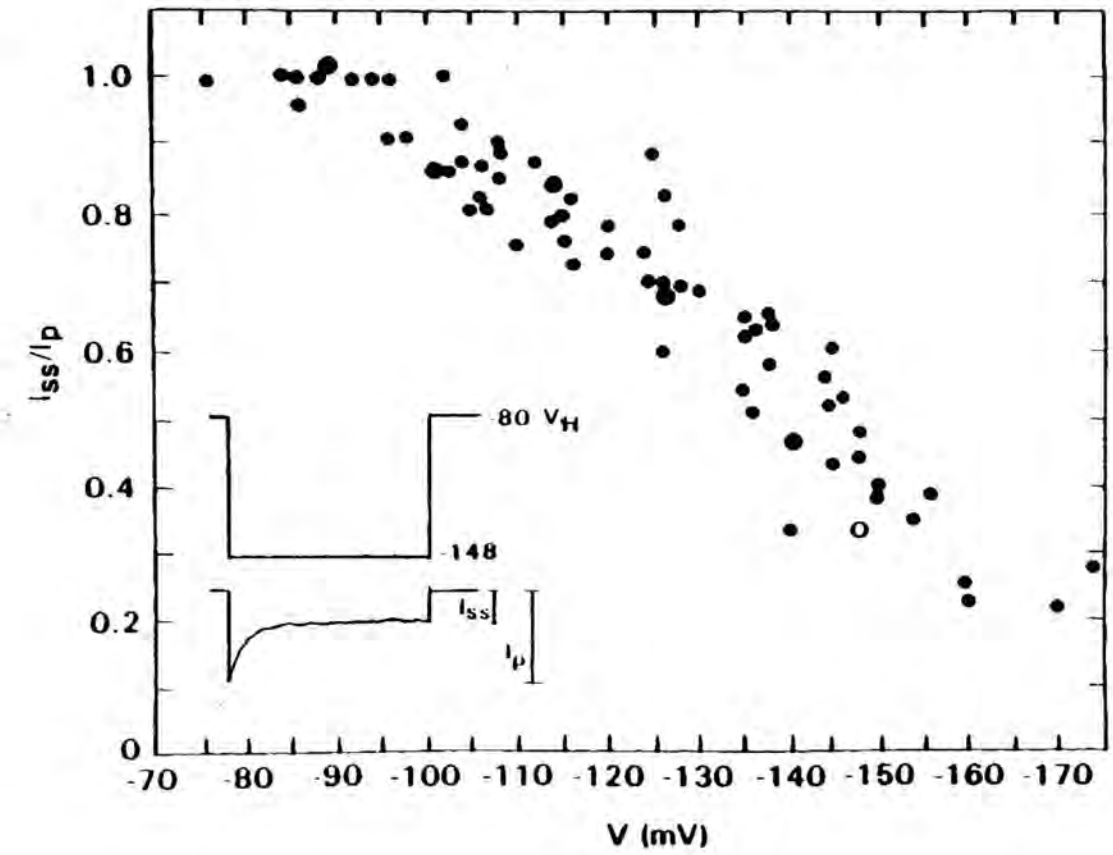

1.0
0.8
0.6
0.4
0.2
0
I_{ss}/I_p
-80 V_H
-148
I_{ss}
I_p
-70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170
V (mV)

Figure 12. A) Semilog plot of inactivating inward current as function of time for voltage steps to -154 (open circle), -145 (solid circle), -136 (square) and -113 (triangle) mV. B) Time constant of decay versus voltage obtained from the cells of Fig. 11. Individual points represent mean time constant from a number of cells (1 to 5) +/- SEM. The two points with no error bar represent data from single cells; 8 different cells were analyzed in all.

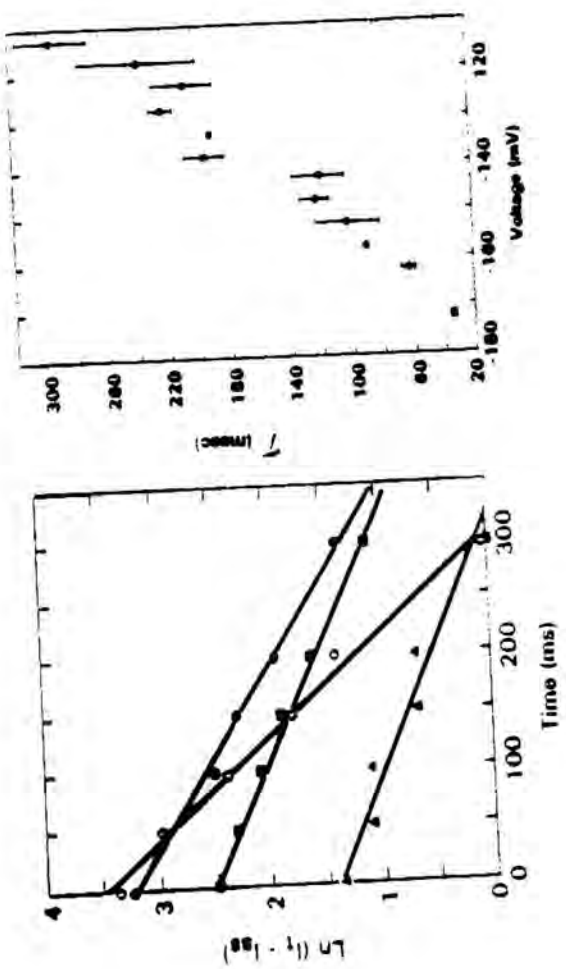
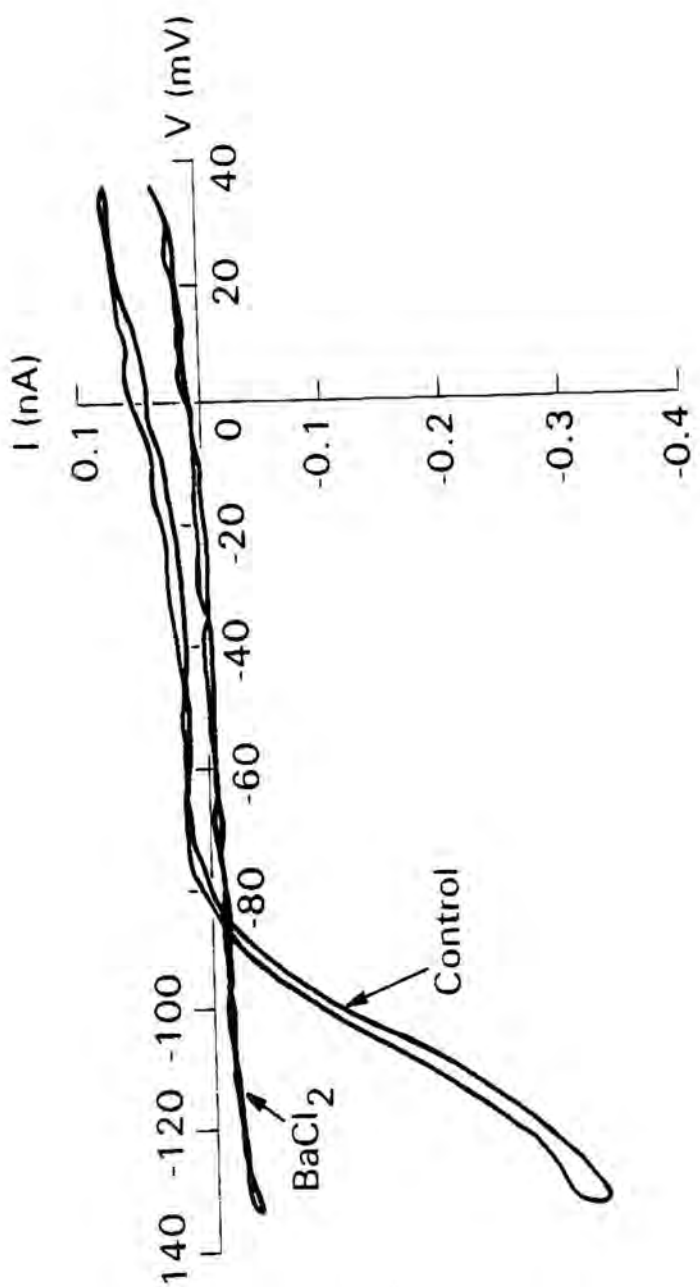
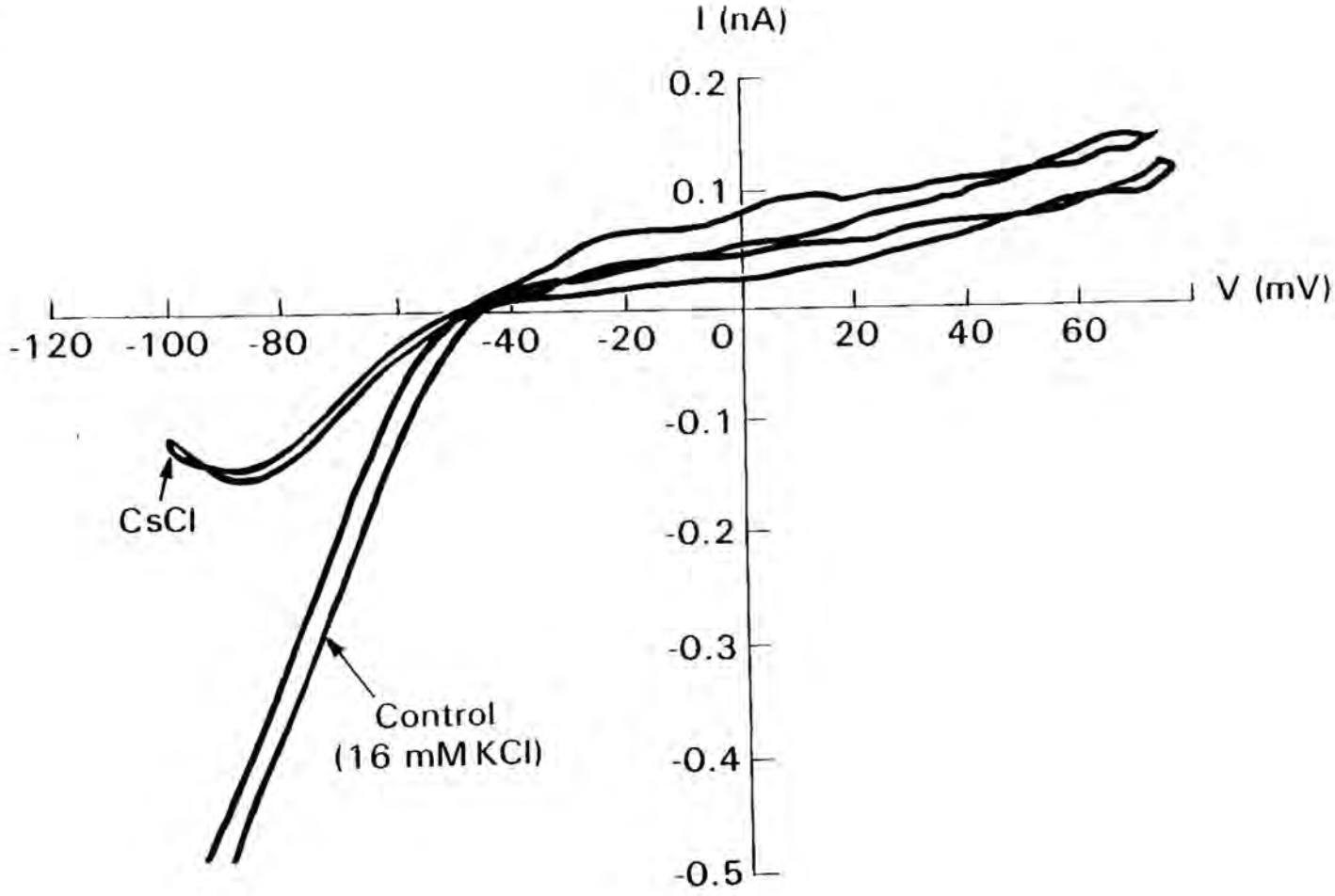

Voltage (mV)
Time (ms)

Figure 13. A) Current/voltage relationships before and after addition of 2.5 mM barium chloride to bath. V_h = -80 mV.
B) Current/voltage relationships before and after addition of 1 mM cesium chloride to bath. $[K]_o$ = 16 mM. V_h = -46 mV.

A


I (nA)
0.1
-140
-120
-100
-80
-60
-40
-20
0
V (mV)
20
40
-0.1
-0.2
-0.3
-0.4
BaCl2
Control

B


I (nA)
0.2
0.1
V (mV)
-120
-100
-80
-40
-20
0
20
40
60
-0.1
-0.2
-0.3
-0.4
-0.5
CsCl
Control
(16 mM KCl)

barium addition became linear with a slope resistance of 3,700 Mohm, a value equal to its resistance in the voltage range -60 to -40 mV before the addition of barium. The zero-current holding potential of this cell shifted from -81 mV to -51 mV. Barium completely eliminated inward rectification in all cells tested (n = 18). The average zero-current holding potential after barium was -49 mV +/- 11.3. Barium block of inward rectification was not voltage-dependent at the barium concentrations and voltages studied. Note that the control and barium-treated I/V curves intersect at -84 mV. The barium-sensitive current therefore reversed direction in -84 mV which suggests that it was carried by K ions (E_k = -85 mV).

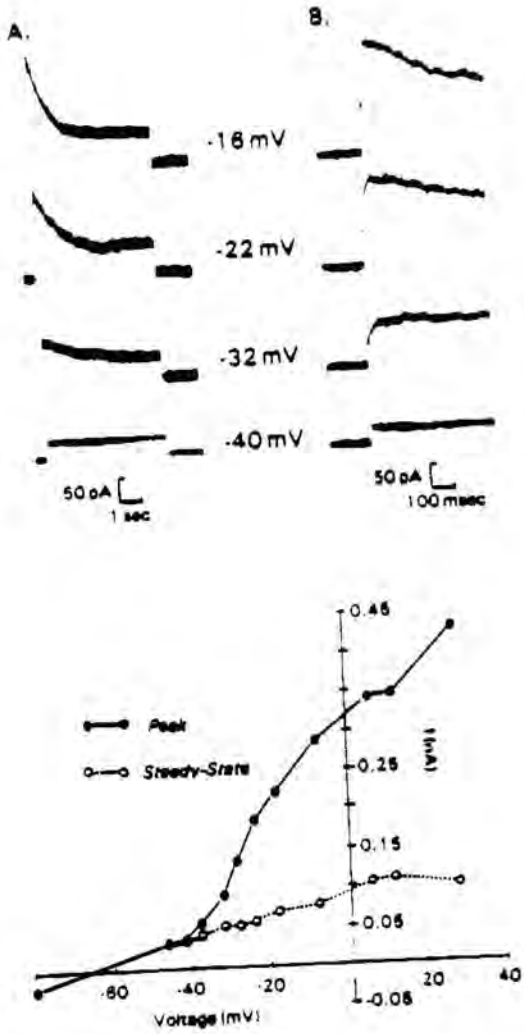
Addition of cesium (1-3 mM), another well known blocker of inward rectification in other tissues (Gay & Stanfield, 1977; Hagiwara and Takahashi, 1974), also reduced the inward rectifying conductance in J774.1 cells (n = 5). Cesium block was voltage-dependent, as shown by the I/V curves of Fig 13b. Block was incomplete at rest and increased with hyperpolarization, producing a region of negative slope resistance.

OUTWARD RECTIFICATION

Current-Voltage Relationship

Outward currents were standardly obtained from cells that had been plated for 1 to 8 hr (>75 cells are presented in this section). Fig. 14a shows current responses of a cell, plated for 3 hr, to voltage steps from a holding voltage of -80 mV. Cells in these studies were held at -80 mV regardless of their actual zero-current potential and a 40 sec interpulse interval was used. Fig. 14a shows that voltage steps positive to -45 mV activated an outward current that declined with time.

Figure 14. Time- and voltage-dependent outward current in a J774.1 cell plated for 3 hours. A) Current responses to series of voltage steps taken from -80 mV. B) Same currents as in Fig. 14a but at 10 times sweep speed. C) Current/voltage relationship of cell in Fig. 14a. Each point is mean of three steps. Solid circles represent current measured 10 msec after start of step, open circles represent the current at end of 5 sec step. Error bars are within the circles. Voltage steps were applied every 40 sec.


A.
B.
-16 mV
-22 mV
-32 mV
-40 mV
50 pA
1 sec
50 pA
100 msec
0.45
0.25
0.15
0.05
(nA)
Peak
Steady-State
-60
-40
-20
20
40
-0.05
Voltage (mV)

The outward current produced by the step to -40 mV peaked at 30 msec. With increasing depolarization, the time to peak was reduced (Fig. 14b). The time course of the rise of the outward current was not systematically investigated because the large capacitative transients obscured very early events. Fig. 14c shows the I/V curve for peak and steady-state currents from the cell in Fig. 14 a & b. It is evident from the steady-state I/V curve that the current completely inactivates.

Ionic Basis of Outward Rectification

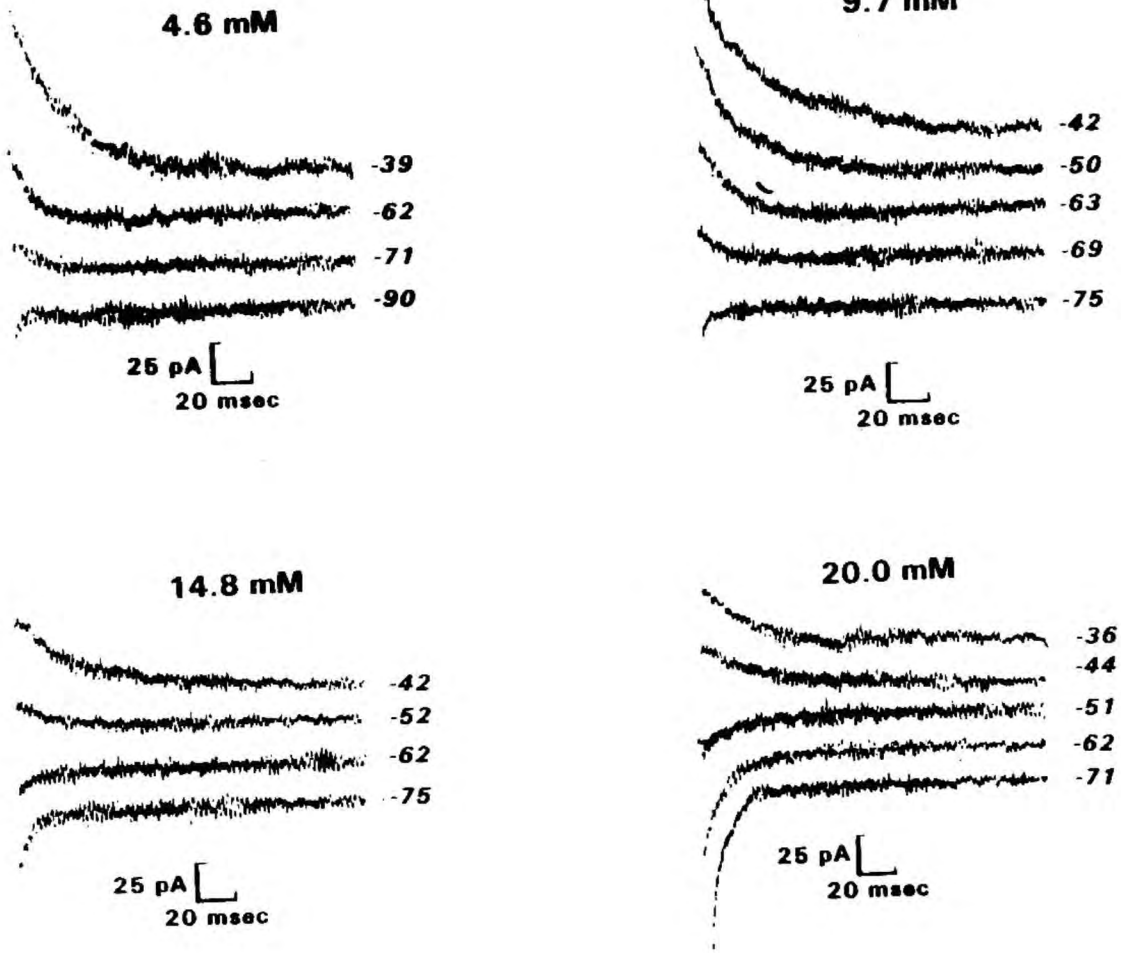
The ionic basis of the outward current was investigated using a two-step pulse protocol. Fig. 15a presents oscilloscope records of tail currents obtained in 4.6, 9.7, 14.8 and 20 mM $[K]_o$. The first pulse was to -10 mV which fully activated the outward conductance. The instantaneous I/V curves (Fig. 15b) were obtained by plotting the amplitude of the tail current (measured 10 msec after the second step minus steady-state current) versus the voltage of the second step. The reversal potential of the outward current shifted to the right as $[K]_o$ increased. Figure 15c shows a plot of the reversal potentials versus log $[K]_o$ obtained by linear regression analysis of the data of Fig. 15b. The slope of the relationship, is -52 mV per tenfold increase in $[K]_o$, ($r = .96$) indicates these currents are carried by potassium. Pooled data of three cells gave a slope of -41 mv per tenfold increase in $[K]_o$.

Conductance-Voltage Relationships

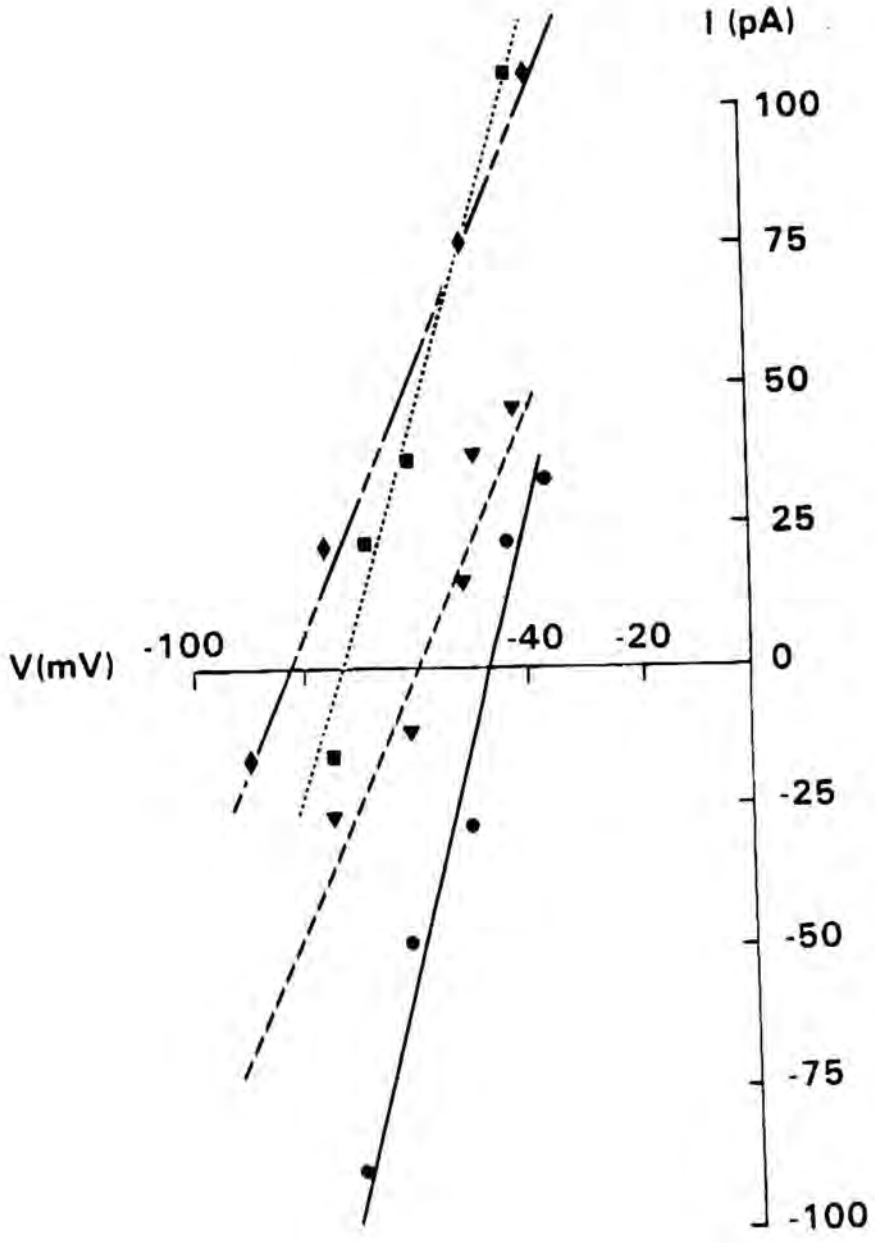
The chord conductance of the outward rectifying current was calculated assuming K^+ to be the predominant charge carrier and E_K to be -85 mV. Fig. 16 presents results from three cells. The rectifying

Figure 15. Tail current analysis of ionic basis of outward rectification. Cell was held at -80 mV and outward conductance was fully activated by a step to -10 mV. A) Current responses of same cell obtained in 4.6, 9.7, 14.8 and 20 mM $[K]_o$. B) Instantaneous I/V relations from the data of Fig. 15a. Symbol, $[K]_o$ mM: Diamond, 4.6; Square, 9.7; Triangle, 14.8; Circle, 20. C) Reversal potential determined from Fig. 15b plotted versus log $[K]_o$. Lines in bothe 15b and c were fit by least squares linear regression ($r>.98$ for both). both).

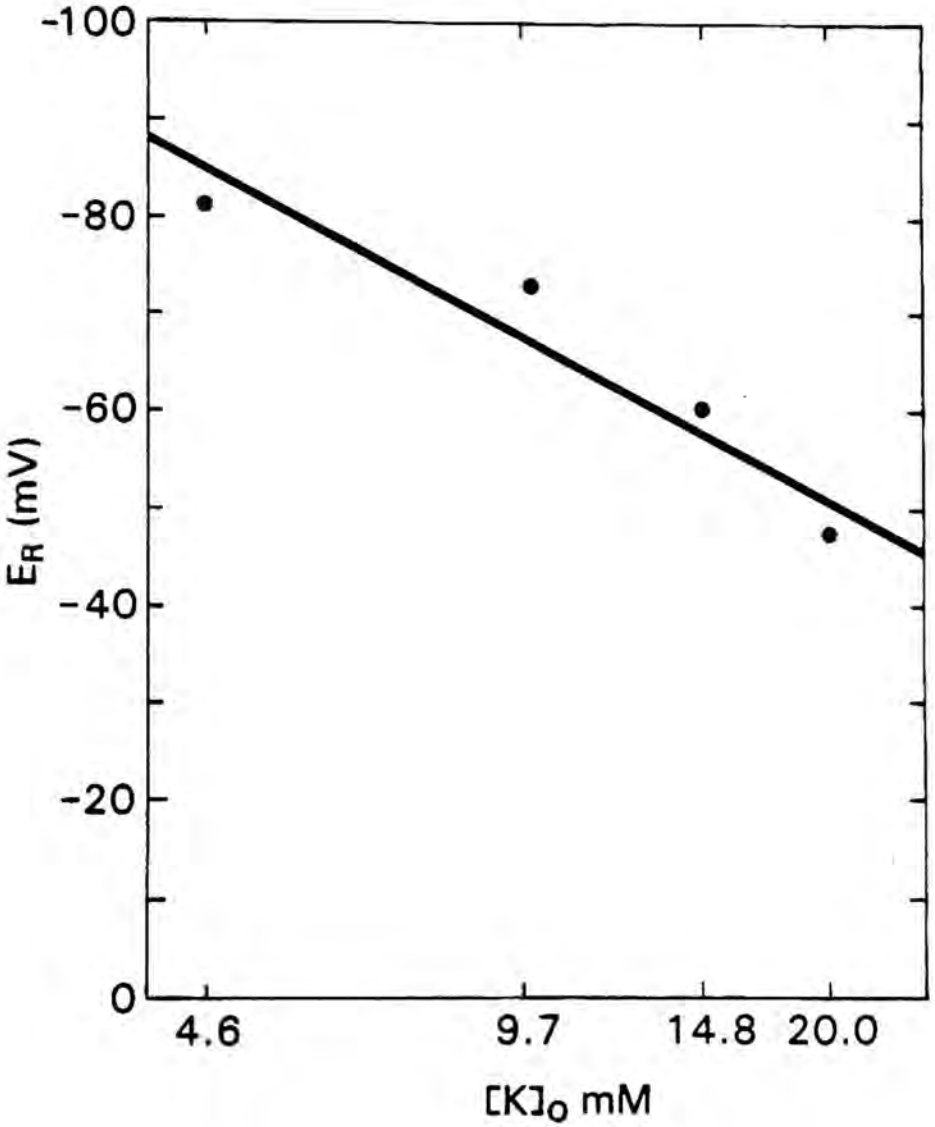
A


4.6 mM
-39
-62
-71
-90
25 pA
20 msec
9.7 mM
-42
-50
-63
-69
-75
25 pA
20 msec
14.8 mM
-42
-52
-62
-75
25 pA
20 msec
20.0 mM
-36
-44
-51
-62
-71
25 pA
20 msec

B



C



current activated at voltages positive to -50 mV and plateaued around -10 mV. The dashed line corresponds to a best fit of the data to the Hodgkin/Huxley equation for the delayed rectifier:

$$G/G_{max} = 1/(1+\exp[(V_m-V_h)/v])^4 \qquad (3)$$

where V_m, V_h and v are defined as in equation 1. The values of V_h and v determined from these data are -44.09 +/- 1.7 and -7.59 +/- 1.02 mV respectively (nonlinear least squares fit, coefficient of multiple determinations = 0.98).

Inactivation

As shown in Fig. 14, the outward currents completely inactivated with time during the voltage step. The time course of the inactivation process was described by a single exponential (Fig. 17a). Fig. 17b shows the time constant of inactivation plotted as a function of voltage for three different cells. At voltages positive to -25 mV, the time constant was essentially independent of voltage, averaging 538 msec +/- 25 (mean +/- S.D.); negative to this potential, inactivation was consistently slower and appeared to have voltage dependence. However, since the amount of inactivating current negative to -25 mV was very small, reliable estimates of the time constants at these voltages could not be made.

The outward rectifying current recovered relatively slowly from inactivation. Paired 250 msec pulses to -10 mv separated by a variable interval were applied. This protocol was repeated three times in each of three cells. The ratio of the peak current elicited by the second pulse (I_2) to that elicited by the first pulse (I_1) is plotted versus the interpulse interval in Fig. 18. The solid line corresponds to a best fit

Figure 16. Chord conductance versus voltage of three freshly plated J774.1 cells, each pulsed to same voltage three times (each point is mean +/- SEM). Each symbol represents a different cell. Conductances were leak subtracted (calculated at -60 mV) and normalized by peak conductance for each cell. Reversal potential was estimated as -85 mV. Dashed line conforms to Eqn. 3.

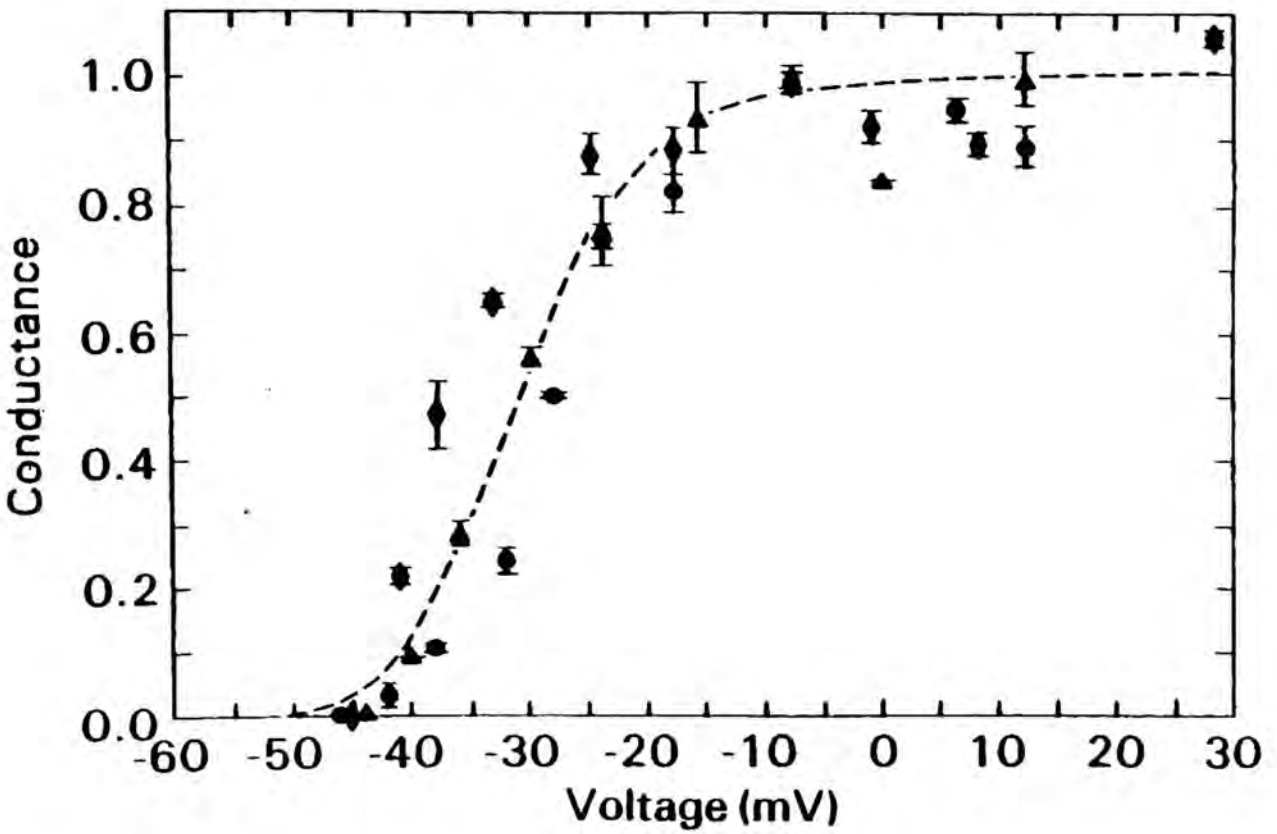
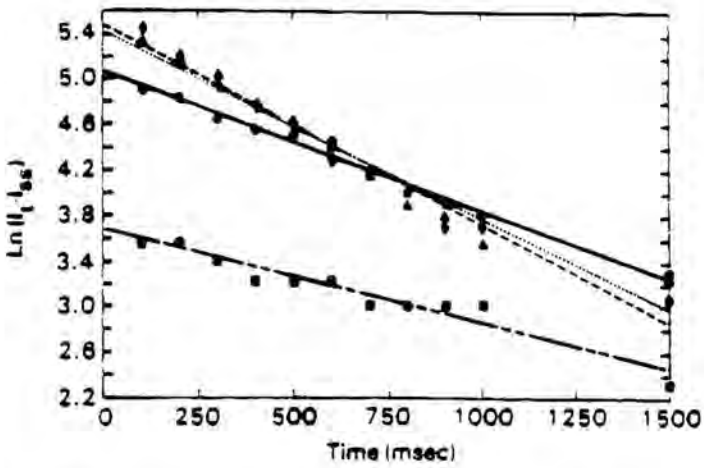

Conductance
1.0
0.8
0.6
0.4
0.2
0.0
-60
-50
-40
-30
-20
-10
0
10
20
30
Voltage (mV)

Figure 17. Time course of inactivation of outward rectification. Steps were taken from holding potential = -80 mV. A) Semi-log plot of inactivating outward current as a function of time for steps to -32 mV (square), -24 mV (circle), -8 mV (triangle) & +12 mV (diamond). B) Time constant of outward current decay versus voltage for three different cells.


5.4
5.0
4.6
4.2
3.8
3.4
3.0
2.6
2.2
Ln (I_t - I_ss)
0
250
500
750
1000
1250
1500
Time (msec)

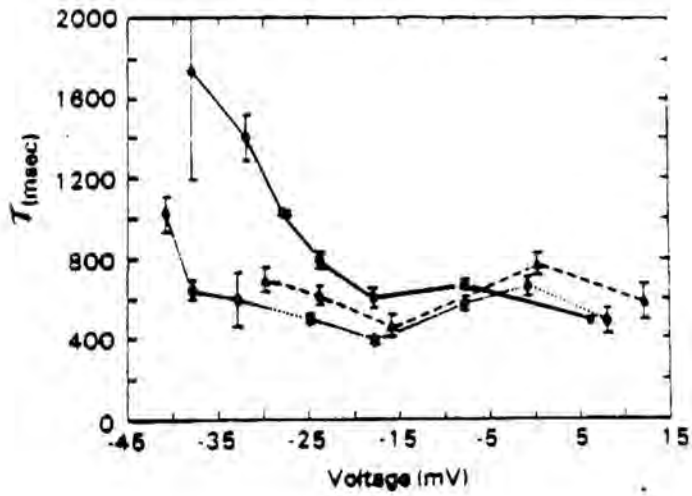
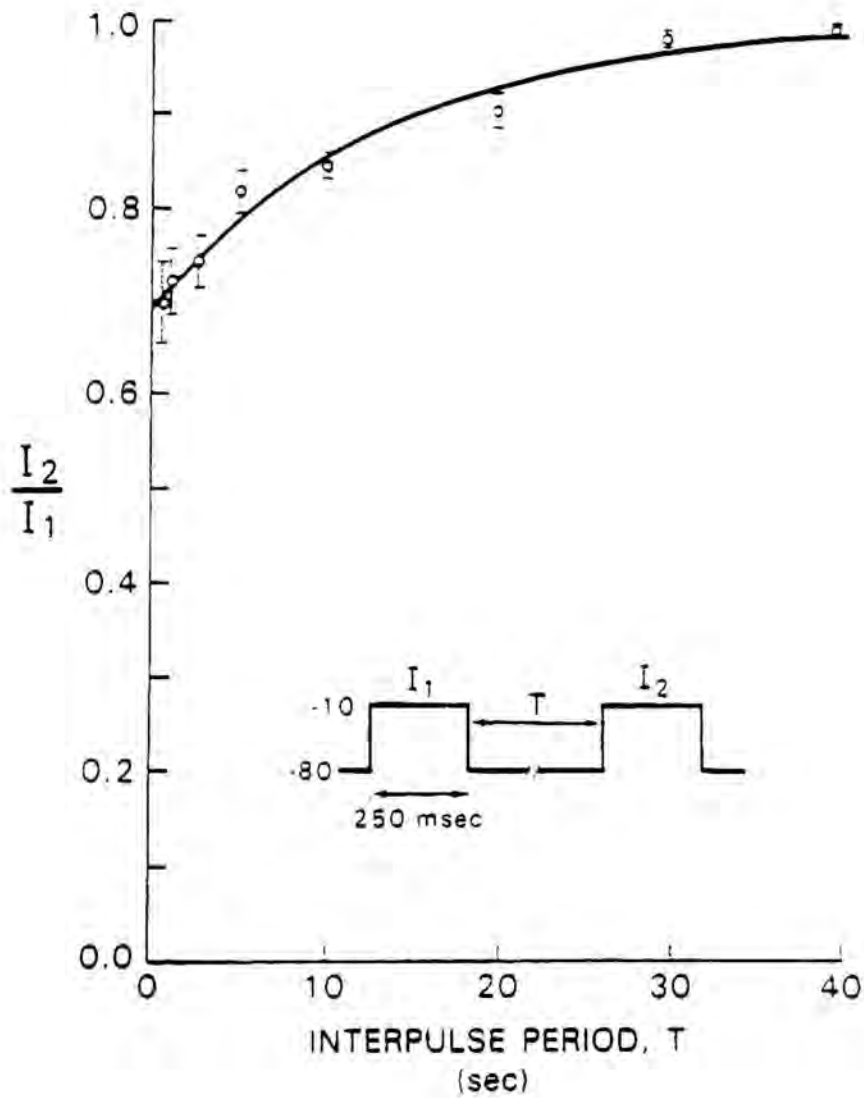

2000
1600
1200
800
400
0
τ (msec)
-45
-35
-25
-15
-5
5
15
Voltage (mV)

Figure 18. Time course of recovery from inactivation of outward rectification. Each point is the mean +/- SEM of three repetitions performed on three cells. The ratio of the peak current during the second pulse (I_2) to that during the first pulse (I_1) is plotted as a function of the interpulse interval. The solid line conforms to:

$$I_2/I_1 = 1 - 0.30\ e^{(t/-13.7)}$$

where t is seconds ($p<.01$).


1.0
0.8
0.6
0.4
0.2
0.0
$\frac{I_2}{I_1}$
0
10
20
30
40
INTERPULSE PERIOD, T
(sec)
I_1
T
I_2
-10
-80
250 msec

of the data to equation (4):

$$I_2/I_1 = 1 - Ke^{-t/\tau} \qquad (4)$$

Where t is in seconds, K is 0.3 +/- 0.012 and τ = 13.7 +/- 1.5 sec (non-linear least squares fit, coefficient of multiple determinations >.999)

Blockers

Complete inhibition of outward rectifying current recorded prior to addition of the blocking agent was obtained with 10 mM 4-aminopyridine (4AP) or 1 mM D600 (methoxyverapamil). Lower concentrations of these agents (1 mM 4AP or 500 uM D600) had only partial blocking action.

Addition of 4AP to the bath produced rapid and extensive vacuolization of J774.1 cells without affecting their passive membrane electrophysiological properties. Recordings obtained from different cells in the same culture dish before and after a 20 minute incubation in 10 mM 4AP showed that the population had the same average RMP and leak conductance (-78.7 versus -78.5 mV and 1.1 versus 1.2 nS respectively). However, the expression of outward rectification decreased from six of seven cells observed prior to addition of 4AP to none of eight after.

STABILITY OF CURRENTS

In J774.1 cells, the activation of outward rectifying current shifted to more negative potentials over the course of the first 10 minutes of a whole-cell recording and peak outward current sometimes increased. After an initial stabilization period, outward current was routinely stable for up to an hour. I/V curves generated with voltage steps immediately after attaining the whole cell conformation and again

after stabilization indicated that peak outward conductance increased and activation shifted. The conductances calculated from the data of 4 different cells pooled before (squares) and again after stabilization (circles) are shown in Fig. 19. The activation curves were fit to eqn. 3 and show a 8 mV shift in V_h to the left; no change was found in v. However, it was apparent that significant shift occurred even as the first I/V curve was obtained.

Ramp-derived I/V curves from an outward rectifying cell obtained before and after stabilization are shown in Fig. 20a. Two principal changes are evident: 1) the inactivating outward current activated at more negative voltages and, 2) a non-inactivating or steady-state outward current apparent at voltages positive to -40 mV decreased. The inactivation characteristics of outward current also changed during stabilization but these changes were difficult to assess because of simultaneous changes in a non-inactivating current evident at voltages positive to -40 mV. Changes in the non-inactivating current were also observed in cells that did not exhibit inactivating K^+ current (Fig. 20b).

Activation curves of the inward conductance exhibited no voltage shifts during whole-cell recording. I/V curves of a long-term adherent cell are shown in Fig. 20b. The most significant change after stabilization was the decrease in conductance at voltages positive to -40 mV. The change in hysteresis in the two curves suggests that inactivation characteristics of the inward rectifier may have changed.

Peak inward conductance was stable with time in most cells not exhibiting inactivating outward current. In cells exhibiting both an inward and an outward current, the peak inward conductance often

Figure 19. Chord conductance versus voltage of four J774.1 cells taken before (squares) and >10 min after stabilization (circles). Conductances were leak subtracted and normalized by the peak for each cell. Reversal potential was assumed to be -85 mV. Lines conform to eqn. 3.

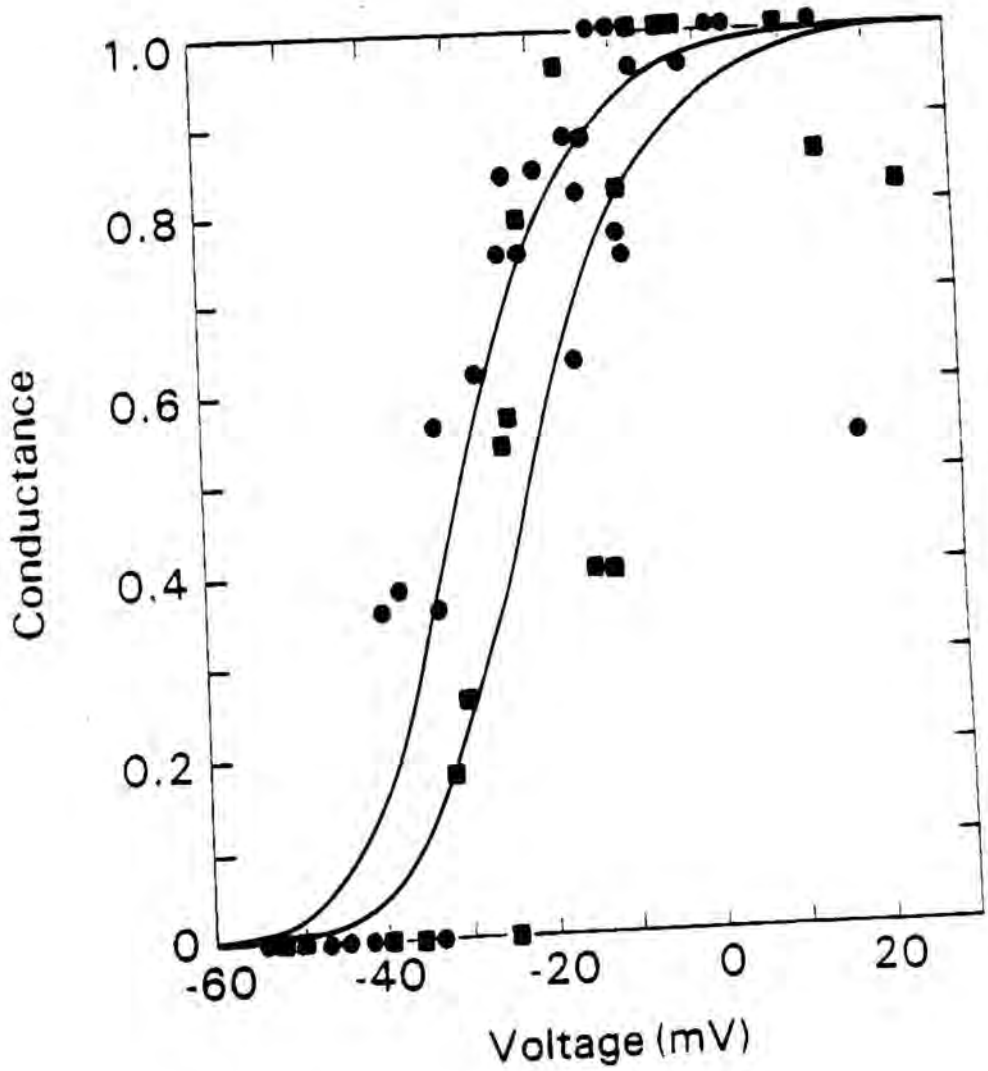
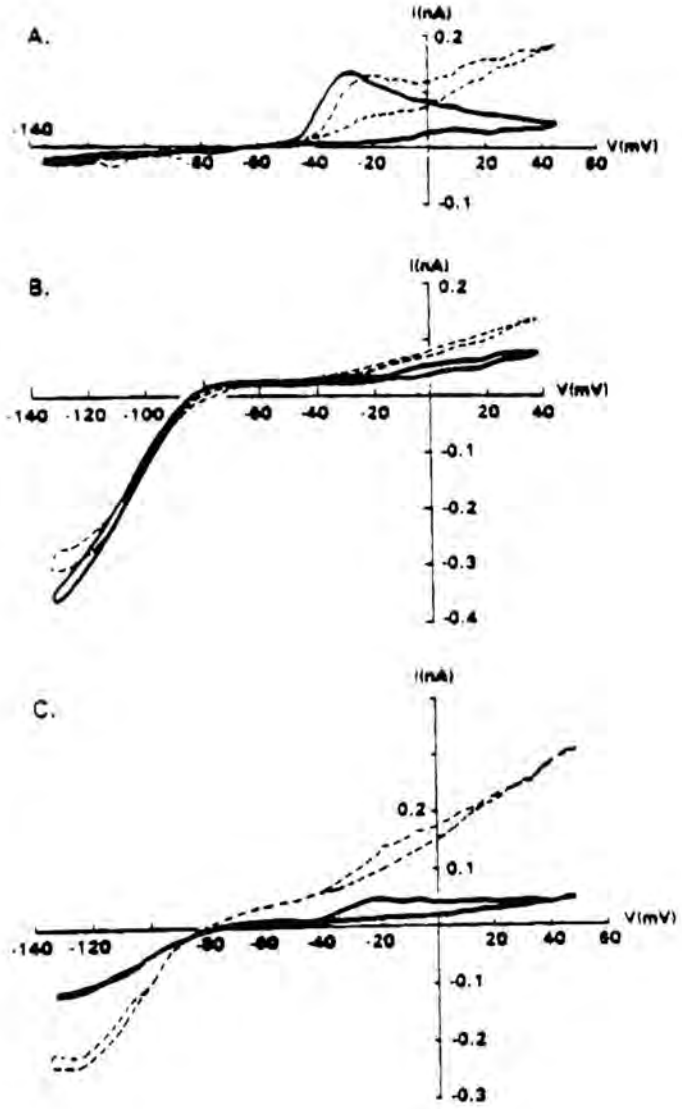

1.0
0.8
0.6
0.4
0.2
0
Conductance
-60
-40
-20
0
20
Voltage (mV)

Figure 20. I/V curves from 3 different cells illustrating the 3 patterns of stabilization observed using whole-cell patch clamp. Dotted lines denote curves obtained immediately after patch rupture. Solid lines denote curves obtained after a stabilization period of 12 minutes; I/V relations were stable thereafter. Curves were generated by voltage ramp. A) Pattern typical of the outward rectifier. B) Pattern typical of long-term adherent cells. C) Patern typical of cells exhibiting both inward and outward rectification.


A.
I(nA)
0.2
-0.1
-140
-80
-60
-40
-20
20
40
60
V(mV)
B.
I(nA)
0.2
-0.1
-0.2
-0.3
-0.4
-140
-120
-100
-80
-40
-20
20
40
V(mV)
C.
I(nA)
0.2
0.1
-0.1
-0.2
-0.3
-140
-120
-80
-60
-40
-20
20
40
60
V(mV)

continued to decline well after outward current had stabilized. Fig. 20c shows the decline of the inward current which occurred over a 12 minute time span; inward rectification eventually completely disappeared. Conductance/voltage curves calculated for the normalized inward current obtained during washout showed no systematic voltage shifts.

DISCUSSION

COMPARISON OF METHODOLOGIES

This study has used both intracellular and patch-clamp microelectrodes to record the membrane potential and current/voltage characteristics of J774.1 cells. Intracelluar electrodes have been used for over forty years and their general validity is well established. On the other hand, certain limitations to the use of intracellular electrodes are also recognized. For the purposes of this study, the most serious of these limitations is the damage to a cell caused by penetrating its membrane with an eletrode. The damage done is a function of the size of the electrode relative to the size of the cell. As a cell decreases in size, the contribution of the region damaged by the electrode relative to the rest of the cell increases and the data obtained are no longer representative of healthy cells. In order to minimize the extent of membrane damage, small electrodes (<0.5 micron tip diameter) are therefore used for intracellular studies of small cells. Unfortunately, the high resistance of such electrodes (>80 Mohm) restricts the types of experiments which can be performed with small cells. In many cases, voltage-clamp experiments are simply not feasible because the electrodes can not pass enough current. Consequently, most intracellular microelectrode studies of macrophages have been performed under current clamp conditions (Gallin, 1984b). Current clamp techniques do not control the capacitative component of the electrical responses and thus the ionic component can not be directly isolated for analysis, although in some cases, it can be inferred (Gallin & Livengood, 1981).

Electrophysiological studies performed with patch microelectrodes have acheived rapid and widespread acceptance (Sakmann & Neher, 1984). These electrodes apparently produce signifigantly less membrane damage than intracellular electrodes. The technique is particularly suited for studies of small cells. Because low resistance electrodes (<10 Mohm) are used, voltage clamp techniques can be used and the cell's ionic currents analayzed.

Openings and closings of single ionic channels can be resolved in the cell-attached and excised patch recording modes (Auerbach & Sachs, 1984; Fenwick, Marty & Neher, 1982). This ability is one of the most attractive features of the technique. Because only the whole-cell mode was used in this study, subsequent comparison of intracellular and patch electrode methods will be restricted to patch electrodes used in the whole-cell mode.

Over the past ten years, intracellular recordings from many types of macrophages and macrophage-like cells have demonstrated three ionic conductances: 1) an inward rectifier, 2) an outward rectifier and 3) a calcium-activated potassium conductance (Gallin, 1984b). Despite considerable effort, reliable recordings have been difficult to obtain because of membrane damage caused by microelectrodes (Gallin & Livengood, 1980). Specifically, most evidence indicates that slow hyperpolarizations reflect an artifact of electrode penetration rather than an ongoing physiological process (Ince et al., 1984) and, as determined by intracellular electrodes, neither the RMP nor the occurrance of inward rectification can be used as absolute indices of cell damage.

The use of patch electrodes generally reduces the membrane damage

produced by intracellular electrodes and thus RMP's are more negative and membrane resistances are higher (Fenwick et al. 1982; Marty & Neher, 1982). But, whole cell patch recording also results in the internal dialysis of the cell with the electrode solution. The ability to control the internal composition of the cell certainly has positive aspects but this may be offset by the loss of important and unidentified intracellular constituents. For example, the dialysis effect allows one to block potassium currents with internal cesium ions or to experimentally set internal ionized calcium concentration with Ca^{2+}-EGTA buffer solutions. On the other hand, a dialysed cell is far from its physiological state and this can alter some ionic currents. As a cell's internal contents exchange with those of the pipette, there is a shift in the voltage sensitivities of some currents and some currents decrease in their amplitude (Fenwick et al., 1982; Fernandez, Fox & Krasne, 1984; Marty & Neher, 1982). Conclusions drawn from whole cell patch experiments must therfore be made with caution.

The results obtained in this study generally support previous findings that the patch clamp technique is better suited to the study of small cell electrophysiology than the conventional intracellular microelectrode technique. As such, conclusions drawn subsequently emphasize the data obtained with patch electrodes.

RESTING MEMBRANE POTENTIAL

The RMP of long-term adherent J774.1 cells determined by intracellular microelectrode current clamp was -51 mV; the distribution of values was broad and unimodal with no obvious peak. The distribution of RMP values from irradiated cells was equally broad but there seemed to

be two subpopulations; one averaged -36 mV and the other -81. Although some depolarized cells in both the normal and irradiated populations exhibited inward rectification, many cells with depolarized RMP's had low resistance, linear I/V relations which suggest penetration-damage. The discrepancy between the distributions of RMP's found in normal and irradiated cells is probably due to the greater size of the irradiated cells. Since larger cells are less susceptible to penetration damage, their RMP's are generally more negative.

The data obtained using the patch clamp technique support the conclusions drawn from intracellular microelectrode studies that healthy long-term adherent J774.1 cells are relatively hyperpolarized and that depolarized RMP's indicate cell damage. In this study, the distribution of zero current values measured by whole-cell voltage clamp was grouped tightly around -77 mV and therefore closely resembled that of the RMP's from the hyperpolarized subpopulation of irradiated cells. No depolarized subpopulations were observed with the patch clamp technique.

Input resistances measured with the patch clamp technique were approximately ten times greater than those determined with intracellular electrodes (1,000 Mohm versus 117 & 95 Mohm for normal and irradiated cells respectively). Since the development of patch clamp recording techniques, it has become evident that small cells typically have input resistances between 1,000 and 10,000 x 10^6 ohm and sometimes greater (Fenwick et al., 1982). Previous estimates of membrane resistance obtained with intracellular electrodes are now recognized as invalid due to shunt conductance pathways introduced into the cell in the damaged membrane surrounding the site of electrode penetration. In large cells, the contribution of this additional conductance path is small relative to

the total cell conductance. As cell size decreases, the contribution of this additional conductance increases. Because patch electrodes seal tightly to the membrane, the conductance across the membrane/electrode seal is small and cells with relatively small total membrane conductances can be studied. Because of this tight seal, recordings in small cells with patch electrodes generally give higher resistances and more negative RMP's than previously obtained with intracellular electrodes (Fenwick et al., 1982; Marty & Neher, 1983).

Results of investigations using the patch clamp technique indicate that the potassium equilibrium potential is the primary determinant of RMP in J774.1 cells. As least one additional permeability must exist however, because the zero-current potentials were systemically positive to that predicted by the Nernst equation for an ideal potassium electrode. This permeability may reflect a shunt conductance across the electrode/cell seal or it may occur normally in unperturbed J774.1 cells. If unperturbed J774 cells were perfect K^+ electrodes, the experimental finding that the slope of their RMP/K^+ relation is 10% less than the ideal 56 mV/decade Nernst relationship suggests that shunt conductance across the seal (which is presumably nonselective) is no more than 10% of the total cell conductance. However, since the cell's permeabilities to sodium and chloride are certainly greater than zero, this is a limiting case estimate and shunt conductance must be less than 10% of the total cell conductance.

With the protocol used to estimate the RMP, it is assumed that the patch clamp technique disturbed neither the cell's ionic gradients nor their membrane permeabilities. However, because the electrode tips used in whole cell patch recording are very wide (usually greater than

several microns), the contents of the electrode exchange with the cytoplasm in only a few minutes. Therefore, in order to obtain a valid estimate of the RMP one must either determine the zero current potential immediately (before signifigant intracellular dialysis has occurred) or use a microelectrode filling solution which has essentially the same composition as the cytoplasm. Both approaches were used in this study. Zero-current potentials were determined within seconds of attaining the whole cell conformation and were stable thereafter, suggesting that the ionic gradients were stable.

The values of RMP's of adherent J774.1 cells measured in this study are very different from the value of -14 mV obtained by Young et al. (1982) using the lipophillic cation TPP^+ to indirectly monitor membrane potential. However, the use of TPP^+ ion to measure RMP's of adherent cells resulted in RMP values around -70 mV (Young, personal communication), which are similar to those obtained in these studies. Therefore, it is likely that the process of adherence produces a rapid (within 30 minutes) increase in the RMP. The change in RMP must involve either an increase in potassium permeability or a decrease in permeability to other ions. Although the inward rectifying conductance is active at the RMP, its expression is not essential for the establishment of negative RMP's since cells soon after plating have -77 mV potentials, but do not exhibit inward rectification.

CONDUCTANCE CHANGES FOLLOWING ADHERENCE

J774.1 cells exhibit both voltage-dependent inward and outward K currents at different times after plating. The outward current appears first, about an hour after plating, and is lost within 12 hr after

plating. The number of cells exhibiting outward current in any given dish was variable, but the time frame of its expression remained consistent. With only one exception (out of 195 cells), outward currents were never observed more than 12 hours after plating. All long-term cultured J774 cells exhibited the inward rectifying potassium conductance whereas it was only seen occasionally in cells plated for less than 3 hours.

Similar inward and outward rectifications have been described in primary cultures of mouse peritoneal macrophages (Gallin & Livengood, 1981; Ypey & Clapham, 1984). However, the time of their appearance and the duration of their expression was different. Ypey and Clapham (1984) reported that the outward current was not present until cells had been cultured under adherent conditions for 12 hours and it lasted for at least 4 days (the duration of the studies). They did not see any inward rectification during the 4-day culture period. Previous work in Dr. Gallin's laboratory has demonstrated that 2-4 week cultured mouse peritoneal macrophages exhibit prominent inward rectification (Gallin & Livengood, 1981). Although these two conductances in primary macrophages are exhibited at later times after plating than in J774.1 cells, the inward rectifier appears after the outward rectifier in both cell types.

The appearance of these two conductances could be the result of one or more of the following processes: 1) the synthesis and subsequent insertion of new channels into the membrane, 2) the insertion of presynthesized channels into the membrane and 3) the modulation of preexisting membrane channels. Since the expression of the outward rectifier in J774.1 cells occurs rapidly, as early as 25 minutes after plating, it is unlikely that <u>de novo</u> protein synthesis is required.

However, inward rectification does not develop until 2-4 hr after plating; therefore, its full expression may involve protein synthesis.

In addition to its efects on potassium conductances, adherence produces other physiological changes in macrophages. For example, Lazdins et al. (1980) have found that glycogen levels in mouse peritoneal macrophages triple within 3 hours after plating. Further, Cohen et al. (1981) demonstrated that 90 minutes after adherence, the release of reactive oxygen metabolites increases in response to phagocytosis or phorbol myristate acetate. Adherent culture conditions decrease the phorbol ester-induced release of superoxide anion (Berton & Gordon, 1981) and alter the membrane transport of several nutrients (Pofit & Strauss, 1977). It is likely that the conductance changes described here are related to some of the functional changes induced by adherence. Other investigators have reported that voltage-dependent conductances are altered during cell development. In particular, peak inward Ca^{2+} current increases in mouse-derived, immunoglobulin secreting hybridomas during development of their secretory capacity (Fukushima et al., 1984b).

These two conductances will have very different effects on a cell. In response to depolarizing stimuli, the inward rectifier shuts off whereas the outward rectifier activates; thus, voltage responses to depolarizing stimuli will be amplified for a cell exhibiting inward rectification but blunted for a cell exhibiting outward rectification. Future studies using pharmacological agents to block these conductances may be useful in determining the relationship between these conductances and cell function.

INWARD RECTIFICATION

The dependence of the inward current in J774.1 cells on both voltage and $([K]_o)^{1/2}$ is similar to that described for the anomalous rectifying potassium conductance in muscle and egg cells (Leech & Stanfield, 1981; Hagiwara & Takahashi, 1974). Inward rectification in J774.1 cells has been demonstrated with both intracellular and patch microelectrodes. Investigations using intracellular microelectrodes performed on long-term adherent cells have demonstrated that inward rectification is characteristic of cells with hyperpolarized RMP's. Since depolarized RMP's indicate cell damage, these studies suggest that the inward rectifier might characterize all healthy J774.1 cells. Data obtained with patch electrodes strongly support this suggestion. A similar inward rectifying current, blocked by barium and enhanced by increasing $[K]_o$, occurs in mouse spleen and thioglycollate-induced peritoneal macrophages cultured for 2-4 weeks (Gallin & Livengood, 1981). Thus, J774.1 cells can serve as a model for determining the physiological role of the inward rectifying current in macrophages.

At this time, no specific function can be attributed to the anomalous rectifier in macrophages or any other cell type. However, the unique relation between the anomalous rectifier and $[K]_o$ is especially pertinent to the macrophage's role as a scavenger, Macrophages are commonly found in sites of dead and dying tissue where $[K]_o$ is locally elevated, the increased conductance may therefore enhance some effector function. In this context, it should be noted that a similar inward rectifying potassium current has recently been noted in a rat basophillic cell line (Ikeda & Weight, 1984).

The conductance/voltage relationship of the inward potassium current in J774.1 cells (equation 1) is similar to that of egg cells (Hagiwara & Takahashi, 1974). However, in J774.1 cells, activation was faster than that in skeletal muscle or starfish eggs (Leech & Stanfield, 1981; Hagiwara et al., 1976), usually being fully activated before the capacitative transient had ended (< 10 msec). In contrast to activation, the voltage dependence of both the rate and extent of inactivation of the inward current in J774.1 cells generally paralleled that of muscle and egg cells. Inactivation followed first-order kinetics with a rate that increased membrane hyperpolarization, as occurs in skeletal muscle (Standen & Stanfield, 1979) and tunicate egg cells (Ohmori, 1978). However, the time constant of decay was longer in J774.1 cells than in muscle and egg cells. As in muscle and egg cells, the degree of inactivation in J774.1 cells increased with voltage, resulting in steady-state I/V relationships with negative-slope resistance regions for potentials negative to -150 mV.

In other cells, inactivation of the inward rectifying potassium conductance results from one or more of three possible mechanisms: 1) a change in driving force due to a potassium redistribution (Almers, 1972a and b) 2) block of the open potassium channel by relatively impermeant ions such as Na^+ (Standen & Stanfield, 1979) and 3) actual channel closure (Sakmann & Trube, 1984). It is unlikely that there is a significant change in the potassium concentration gradient across the J774.1 membrane since there is no evidence to suggest that ions on either side of the membrane are restrcited in any way from access to the large concentration sinks provided internally by the patch electrode and externally by the bathing medium. The question of open channel block

versus true channel closure would best be approached by single-channel techniques rather than the whole-cell method used in this study. Since significant current decay (up to 40%) was seen at potentials where Na^+ block is not thought to predominate in other cells (Standen & Stanfield, 1979), channel closure is probably the predominant mechanism in J774.1 cells in the voltage range studied in these experiments.

OUTWARD RECTIFICATION

The inactivating outward potassium current of J774.1 cells is very similar to that of peritoneal macrophages and T lymphocytes (Ypey & Clapham, 1984; Cahalan et al., 1985; Fukushima, Hagiwara & Henkart, 1984). The voltage dependence of activation of the outward conductance in J774.1 cells could be described by Hodgkin/Huxley-type kinetics, although the fit was not significantly altered by fourth-power or first-power exponents ($p<.01$ for either fit). The values of V_h and v determined in this study (-44 and -7.6 mV respectively) are quite close to those reported in T lymphocytes (-47.4 and -6.8 mV) by Cahalan et al. (1985).

The time course of inactivation of the outward current followed a single exponential at all voltages studied, and showed no systematic voltage dependence at voltages positive to -25 mV. In two cells, the rate of decay clearly decreased at voltages negative to -25 mV, but the measurement error was too large to allow accurate estimates for the whole population. These results are similar to those of Cahalan et al. (1984) in human T lymphocytes.

The inactivating outward current in J774.1 cells was completely blocked by 10 mM 4AP. This concentration is similar to the effective

concentrations reported in mouse peritoneal macrophages (5 mM 4AP; Ypey & Clapham, 1984) and human T lymphocytes (10 mM 4AP; DeCoursey et al., 1984).

The sensitivity of the outward current to D600, an agent which blocks voltage-dependent calcium channels suggests that the conductance might be activated by the influx of calcium. Studies of excised patches from human macrophages have demonstrated large conductance calcium- and voltage-dependent K^+ channels (Gallin, 1984). If similar channels exist in J774.1 cells (and were to play a role in these outward currents), the internal ionized calcium would have have to be on the order of 10^{-5} M to produce the voltage dependence reported here. This is unlikely since the electrode contains 1.1 mM EGTA and inward (calcium) currents were not seen in these cells. Furthermore, the time course of activation and inactivation of the current are inconsistent with a calcium-activated potassium conductance.

STABILITY OF CURRENTS

The zero-current potential determined immediately (within seconds) after obtaining a whole cell patch recording usually remained stable for an hour or longer. However, I/V curves obtained after the first 10 minutes of recording were different from those obtained initially. After stabilization, all cells showed a decrease in steady-state current at voltages positive to -40 mV and the inactivating outward current activated at more negative voltages. The estimate of a 8 mV shift in the activation of the outward current is most likely an underestimate because of the time (3-4 minutes) required to obtain the initial I/V relationship. However, the time course and magnitude of the

shift in J774.1 cells is similar to that seen in lymphocytes by Fukushima, Hagiwara & Henkart (1984).

In this study, activation of the outward rectifier shifted to more negative voltages and peak outward conductance increased under whole-cell recording conditions. In contrast, activation of the inward rectifier was stable but peak inward conductance decreased. Interestingly, washout of the inward rectifying conductance was primarily seen in cells exhibiting both inward and outward rectification, much more so than in cells exhibiting only inward rectification. The decline in peak inward conductance continued after the outward current had stabilized.

Two general mechanisms have been proposed to explain the conductance changes apparently produced by patch electrodes: 1) dilution or loss of intracellular modulatory substances and 2) alterations of membrane surface charge (Fernandez et al., 1984). The relative contribution of these two mechanisms to the changes observed in the outward cannot be determined form the present data. However, the normalized inward conductance/voltage curve did not shift during washout of the inward current, it is unlikely that a change in surface charge is responsible for the washout of inward current in freshly plated cells.

GENERAL CONCLUSIONS

Two techniques have been used in this study to characterize the electrophysiological properties of the J774.1 macrophage-like cell line (Ralph & Nakoinz, 1975). Results obtained with intracellular electrodes indicate that long-term adherent J774.1 cells exhibit broad distributions of RMP's and R_m's as well as a variable expression of membrane ionic

conductances. Three currents were observed with intracellular electrodes: 1) an inward rectification expressed by cells with relatively negative RMP's that was most likely due to a potassium conductance which activates with hyperpolarization, 2) slow hypolarizations were occaisionally seen, most often in relatively depolarized cells, that were most likely due to a Ca^{2+}-activated potassium conductance and 3) an outward rectification whose ionic basis was not investigated. Results obtained with patch electrodes indicated that long-term adherent J774.1 cells exhibit a tight distribution of relatively hyperpolarized RMP's and R_m's. All long-term adherent J774.1 cells exhibited inward rectification, no evodence was found for other conductances in such cells using the patch clamp technique.

As J774.1 cells were transfered from suspension to adherent culture, two time- and voltage-dependent potassium currents were sequentially expressed. The two had distinct voltage sensitivities: one activated at voltages positive to -40 mV and the other activated at voltages negative to -60 mV. The former is similar to the outward rectifying potassium conductance described in freshly plated mouse resident pertioneal macrophages (Ypey & Clapham, 1984). The latter is similar to the inward rectifying potassium conductance described in long-term cultures of mouse spleen and thioglycolate-elicited macrophages (Gallin, 1981; Gallin & Livengood, 1981).

It is perhaps significant that these cells are derived from a type of tumor which can grow either as an ascites or in a solid form. The expression of these currents in J774.1 cells may reflect some change in their neoplastic properties. However, these currents are also found in normal macrophages and adherence changes many of the functional

properties of normal macrophages as well. The ability to select for genetic variants of the parental J774 line suggests that these cells may serve as a model system for the investigation of the role of voltage-dependent ion conductances in macrophage function.

APPENDICES

I CALCULATION OF TIME CONSTANTS

A passive membrane is standardly modelled as a circuit consisting of a resistor and a capacitor in parallel. Total current is the sum of flow through each element of the circuit.

$$I(t) = I_r(t) + I_c(t) \quad (1)$$

where $I(t)$ is total current, $I_r(t)$ and $I_c(t)$ are the current components through the resistor and the capacitor respectively as functions of time. From Ohm's Law, the current through the resistive element is given by:

$$Ir(t) = V(t)/R \quad (2)$$

where $V(t)$ is the voltage drop across the circuit as a function of time and R is the value of the resistor. The capacitive current is given by:

$$I_c(t) = C(dV/dt) \quad (3)$$

where C is the value of the capacitor and dV/dt is the change in voltage with time. Combining equations 1, 2 and 3 produces:

$$I(t) = V(t)/R + C(dV/dt) \quad (4)$$

Under current clamp, the amount of current injected is a constant, R equals membrane resistance (R_m) and C equals membrane capacitance (C_m). With the constraint that I is independent of time, the solution to equation 4 is given by the steady-state voltage (V_o) minus an exponential term:

$$V(t) = V_o - V_o(e^{-t/\tau})$$

which can be rearranged to yield:

$$V_o - V(t) = V_o(e^{-t/\tau}) \quad (5)$$

where

$$V_o = I \times R_m \qquad (5a)$$

$$\tau = R_m \times C_m \qquad (5b)$$

Thus, voltage changes as an exponential function of time. As membrane capacitance charges, current is carried increasingly by the resistive element, thus voltage asymptotically approaches a steady-state value, $V_o = I \times R_m$ (eqn. 5a). Plotting the logarithm of the time-dependent voltage ($V_o - V(t)$) versus time generates a straight line with a slope equal to the time constant for charging the membrane capacitance, $\tau = R_m \times C_m$ (eqn. 5b). Knowing the current injected (I) and the steady-state voltage (V_o), one can calculate membrane resistance ($R_m = V_o/I$). Membrane capacitance is calculated from the ratio of the time constant to membrane resistance ($C_m = \ /R_m$).

Membrane capacitance is the product of specific capacitance times total membrane area. Using a well-established value for specific capacitance ($C = 1$ uF/cm^2), one can then determine the cell's surface area (A) from its total capacitance.

$$A = C_m/C \quad (7)$$

The membrane specific resistance (R_s) can then be calculated from the total membrane resistance divided by surface area.

$$R_s = R_m/A \quad (8)$$

II CALCULATION OF CONDUCTANCE

A cell's conductance G(V), equal to the reciprocal of resistance, can be calculated for any two voltages V_1 & V_2 as the slope of the cell's I/V relationship between the two.

$$G(V_1,V_2) = (IV_1 - I_2)/(V_1 - V_2) \quad (1)$$

However, conductance for a specific ion is calculated by dividing the

current carried by that ion by the ion's driving force (the difference between the cell's membrane potential and the ion's equilibrium potential). The equilibrium potential of an ion is that voltage at which the current carried by the ion is zero. The equilibrium potential (E) of an ion is given by the Nernst equation:

$$E = -(RT/nF) \times \ln ([]_i/[]_o) \qquad (2)$$

where R = the gas constant, T = temperature, n = the valence of the ion, F = Faraday's constant and $[]_i$ & $[]_o$ refer to the intracellular and extracellular concentrations of the ion respectively; for a monovalent ion at room temperature RT/nF = 56 mV.

Since current flow at the equilibrium potential (E_i) is zero by definition, it is only necessary to measure current at one potential ($Vm_)$. Thus, the conductance of a specific ion ($G_i(V_m)$) is given by:

$$G_i(V) = I(V)/(E_i - V_m) \qquad (3)$$

The current recorded at any given potential is the result of a combination of both passive and active components, that is, both linear (leak) and voltage-dependent conductances. In order to analyze the voltage-dependence of a particular ionic conductance, the linear component must be subtracted from the total current. In practice, a voltage range is empirically defined which best represents the cell's passive elements and leak currwnt (I_L) is extrapolated from this range. In the present study, leak was estimated from the steady-state currents measured at -40 and -30 mV. I/V relations were directly generated on line using a a voltage ramp and a X-Y recorder; a line extrapolating leak current was drawn on the graph and both leak and total current were recorded as a function of voltage. Chord conductance was calculated by dividing the amount of leak-subtracted current at each potential by the

ion's driving force. Thus the equation used in this study to calculate chord conductance for potassium is given by:

$$G_k(V) = (I(V) - (I_L)/(E_k - V_m) \quad (4)$$

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