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Identification of [^{3}H]P1075 binding sites and P1075-activated K^+ currents in ovine choroid plexus cells

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Abstract

This study examined the pharmacological characteristics of binding sites for the potent K^+ channel opener [^{3}H]P1075, as well as the functional effects of P1075 on ionic currents and membrane potential, in ovine choroid plexus (OCP) cells. [^{3}H]P1075 bound to OCP cells with a K_d of 26 ± 4 nM and a B_{max} of $10\,400 \pm 480$ sites/cell. Labelled sites were stereoselective and inhibited by potassium channel openers with a rank order of potency: P1075 > BMS-182264, ((4-[[9cyanoimino)[(1,2,2-trimethylpropyl)amino]-methyl]amino]benzonitrile) > pinacidil ≫ nicorandil > diazoxide. The K_{ATP} channel antagonist glyburide inhibited [^{3}H]P1075 binding with a K_i of 2 μM. The presence of K_{ATP} channels on OCP cells was examined by patch clamp and fluorescent (membrane-potential sensitive dye) techniques. In some cells, P1075 activated an outward potassium current which was blocked by glyburide. P1075 produced a glyburide-sensitive, concentration-dependent, hyperpolarization of OCP cells. Levcromakalim hyperpolarized more strongly than its 3*R*,4*S* enantiomer, BRL 38226 ((3*R-trans*)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-2*H*-1-benzopyran-6-carbonitrile) indicating a stereoselective interaction. These data indicate that epithelial OCP cells contain glyburide-sensitive K_{ATP} channels. © 1998 Elsevier Science B.V.

Keywords: K^+ channel opener; Choroid plexus; P1075; Glyburide; Levcromakalim; K_{ATP} channel; Membrane potential

1. Introduction

Adenosine 5′-triphosphate sensitive K^+ (K_{ATP}) channels are regulated by intracellular ATP and modulated by nucleotide di- and triphosphates (Terzic et al., 1994). A structurally diverse class of drugs referred to as potassium channel openers have been shown to activate K_{ATP} channels (Quast and Cook, 1989). The potent potassium channel opener P1075 has been radiolabelled and shown to label sites in intact aortic tissue (Bray and Quast, 1992), cardiac myocytes and cultured smooth muscle cells (Dickinson et al., 1993). The present study describes the use of [^{3}H]P1075 to characterize sites on epithelial cells derived from ovine choroid plexus.

Epithelial cells of the choroid plexus transport K^+ ions across their apical and basolateral surfaces and thereby regulate cerebrospinal fluid K^+ concentration. The presence of inward rectifier and delayed rectifier potassium currents, as well as a cAMP-activated chloride current, have been described in rat choroid plexus (Kibble et al., 1996; Kotera and Brown, 1994) but no information exists regarding the presence of K_{ATP} channels in choroid plexus cells. Since potassium channel opener binding sites are generally thought to be associated with sulfonylurea receptors (Bernardi et al., 1992) or associated inward rectifier proteins (Terzic et al., 1994; Inagaki et al., 1995), we were interested in establishing the presence of functional K_{ATP} channels in these cells. To this end, we used patch clamp techniques and fluorescent imaging with the membrane potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] to demonstrate the pres-

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ence of a potassium channel opener-activated, glyburide-sensitive, hyperpolarizing outward potassium current.

2. Materials and methods

2.1. Ovine choroid plexus (OCP) cells

OCP cells, obtained from ATCC, Rockville, MD (cat # 1700), were cultured in Eagles minimal essential media supplemented with non-essential amino acids, Earle's balanced salt solution, and 10% fetal bovine serum at 37°C with 5% CO_2. Cells were sub-cultured three times per week using 0.25% trypsin and EDTA and cells were harvested for binding assays using trypsin/EDTA.

2.2. [^{3}H]P1075 binding

Assays were conducted in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered-physiological salt solution (HEPES-PSS) using 12×75 polypropylene tubes. The incubation mixture contained 15 nM [^{3}H]P1075 (61Ci mmol^{-1}) and displacing drug, or P1075 (10 μM) to define non-specific binding. The binding reaction was initiated by the addition of 200 μl of OCP cells (7×10^5). The tubes were incubated at 22°C for 60 min. Bound and free radioligand were separated by filtration through a GF/D glass fiber filter on a Brandel™ cell harvester with 3×4 ml washes of 135 mM NaCl, 10 mM HEPES, pH 7.4 at 4°C. The filters were transferred to 6-ml plastic scintillation vials, soaked overnight in 5 ml of scintillation fluid (Packard's Opti-fluor) and counted in a Packard liquid scintillation counter at 60% efficiency. Saturation and competition experiments were analyzed by iterative fitting of data to a one-binding site model. K_i values were calculated from IC_{50} values by use of the equation by Cheng and Prusoff (1973).

2.3. Patch clamp techniques

Ionic currents were recorded from OCP cells using the perforated patch technique (Horn and Marty, 1988) using patch electrodes fabricated from borosilicate glass (2–5 MΩ). Voltage ramps from -120 to 60 mV (6.4 mV s^{-1}) were applied from a holding potential of -40 mV using pClamp 6 software (Axon Instruments) run on a Compaq computer via a Digidata 1200 interface (Axon Instruments) and List EPC7 amplifier. Resultant currents were filtered at 3 kHz using an 8-pole Bessel filter (Frequency Devices) and stored on computer for later analysis. The pipette solution contained (mM): KCl, 55; K_2SO_4, 75; $MgCl_2$, 5; HEPES, 10; pH 7.3 with KOH; 100–150 mg ml^{-1} nystatin. The external bathing solution was a HEPES-buffered PSS of the following composition (mM): KCl, 4.6; NaCl, 140; $MgCl_2$, 1; $CaCl_2$, 1.5; HEPES, 10; glucose, 10; pH 7.3 with NaOH. Experiments were performed at $\sim$33°C. Drugs were added to the external bathing solution that superfused the recording chamber.

2.4. Fluorescence measurements

OCP cells, that were cultured in black-sided clear bottomed 96-well plates (Polyfiltronics, Rockland, MA), were washed two times with HEPES-buffered Earles's balanced salt solution and incubated for 15 min at 37°C in media containing 5 μM $DiBAC_4(3)$. The total volume of incubants was 180 μl. Antagonists were pre-incubated with cells for 15 min prior to potassium channel opener addition. The microtitre plate was placed in a fluorescence-imaging plate reader (FLIPR™; Molecular Devices) that determined cell-associated fluorescence simultaneously in all wells of a 96-well microtitre plate maintained at 37°C. An additional plate was prepared which contained drugs dissolved in $DiBAC_4(3)$ solution. Drugs (20 μl) were pipetted from this plate to the cell plate and the contents mixed. The OCP cell monolayer was excited with laser light (488 nm) and cell-associated fluorescence was measured every min for a period of 25 min using FLIPR™. Flourescence was recorded as arbitrary fluorescence units and normalized to the fluorescence response to 30 mM KCl (100%). Experimental results represent mean data from multiple wells (usually 12).

2.5. Drugs

[^{3}H]P1075 was prepared in house by catalytic tritiation of an olefinic precursor. Two tritium atoms were incorporated/P1075 molecule and the resultant radioligand had a current specific activity of 51–61 Ci/mmol. Glyburide and diazoxide were obtained from Research Biochemical, Natick, MA. $DiBAC_4(3)$ was purchased from Molecular Probes, Eugene, OR. P1075, pinacidil, BMS-182264 ((4-[[9cyanoimino)[(1,2,2-trimethylpropyl)amino]-methyl]-amino]benzonitrile), BMS-180447 ((3*S-trans*)-*N*′-Cyano-*N*-(6-cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-4-yl)-*N*′-phenylguanidine), levcromakalim and BRL-38226, (3*R-trans*)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-2*H*-1-benzopyran-6-carbonitrile, were synthesized in house. Nicorandil was a gift from Upjohn, Kalamazoo, MI.

3. Results

3.1. Characterization of [^{3}H]P1075 binding sites on ovine choroid plexus cells

[^{3}H]P1075 bound to OCP cells in a saturable and specific manner, yielding a K_d of 26 ± 4 nM ($n = 3$). The B_{max} was 17.3 ± 0.8 fmol per 10^6 cells, equivalent to $10\,400 \pm 480$ sites/cell. Specific binding (defined with 10 μM P1075) at 14 nM [^{3}H]P1075 represented 40–50% of

Table 1
Inhibition constants of drugs for [^{3}H]P1075 binding sites on OCP cells

Compound	K_i (nM)	n
P1075	34 ± 13	6
Pinacidil	340 ± 200	6
BMS-182264	100 ± 20	5
Levcromakalim	520 ± 120	4
BRL 38226	44 000 ± 19 000	3
BMS-180447	12 700 ± 5900	7
Diazoxide	55 000 ± 14 000	4
Nicorandil	10 900 ± 4800	5
Glyburide	2000 ± 300	3

IC_{50} values were obtained in competition with 15 nM [^{3}H]P1075, and K_is calculated from the equation of Cheng and Prusoff (1973). Results show the mean (± S.E.) of the stated number of experiments.

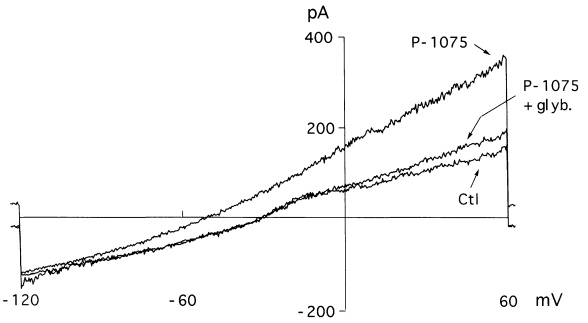

Fig. 1. Effect of P1075 on whole cell currents recorded from OCP cells. Voltage ramps were applied from −120 to 60 mV (6.4 mV s^{-1}) from a holding potential of −40 mV (upper figure). Current was recorded in the absence of drugs (control: Ctl) or presence of P1075 (1 μM) or P1075 and glyburide (glyb. 1 μM).

total binding. The pharmacological characteristics of [^{3}H]P1075 sites on OCP cells were determined by competition experiments. P1075 was the most potent inhibitor with an inhibition constant (K_i = 34 nM) which was similar to the K_d determined for the radioligand (Table 1). Pinacidil was 10-fold less potent and its analog BMS-182264 ((4-[[9cyanoimino][(1,2,2-trimethylpropyl)amino]-methyl]amino]benzonitrile), had a K_i of 100 nM. The binding sites demonstrated stereoselectivity for cromakalim since the 3*S*,4*R* enantiomer, levcromakalim, was 85-fold more potent than its 3*R*,4*S* enantiomer BRL 38226 ((3*R-trans*)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-2*H*-1-benzopyran-6-carbonitrile). BMS-180447, ((3*S-trans*)-*N*′-Cyano-*N*-(6-cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-4-yl)-*N*′-phenyl-guanidine), a structurally related benzopyran potassium channel opener, was 24-fold less active than levcromakalim and its K_i was similar to that of the less potent potassium channel opener nicorandil. Diazoxide had the lowest potency, and its calculated K_i value was 55 μM. The antagonist glyburide inhibited [^{3}H]P1075 binding to OCP cells in a concentration-dependent manner and its calculated K_i was 2 ± 0.3 μM (Table 1). Competition curves of glyburide had a mean slope factor of 0.84 ± 0.05.

3.2. Demonstration of K_{ATP} current in OCP cells

Under control conditions, application of a voltage ramp from −120 to 60 mV resulted in the activation of an outward current at voltages positive to ∼ −40 mV (Fig. 1); the presence of a delayed rectifier current was confirmed in a separate series of experiments employing brief (200 ms) depolarizing voltage steps (data not shown). Application of the potassium channel opener P1075 at 1 μM, a concentration expected to evoke a robust response, produced an increase in outward current (50–200 pA at +60 mV) in some cells (4 of 16 cells; Fig. 1). On the average, the currents measured following activation with P1075 intersected with control currents at voltages close to EK, indicating that P1075 evoked a potassium current. Moreover, the P1075-induced increase was reversed by the subsequent application of glyburide (1 μM; Fig. 1). Most of the cells that failed to respond to P1075 were further challenged with dinitrophenol (200 μM) in an attempt to indirectly activate ATP-regulated potassium channels (Findlay, 1993; Alekseev et al., 1997). This procedure failed to evoke an increase in outward current in any of the cells tested (n = 10). Thus, cultured OCP cells appear to express ATP-regulated potassium channels. However, there is a high degree of cell-to-cell variability in the level of functional expression.

3.3. Potassium channel opener-mediated effects on OCP membrane potential

Fig. 2 shows the fluorescence response of OCP cells which were depolarized with 30 mM KCl or hyperpolarized with potassium channel openers. KCl produced a time-dependent increase in fluorescence which reached maximum after 20 min; this response was normalized to

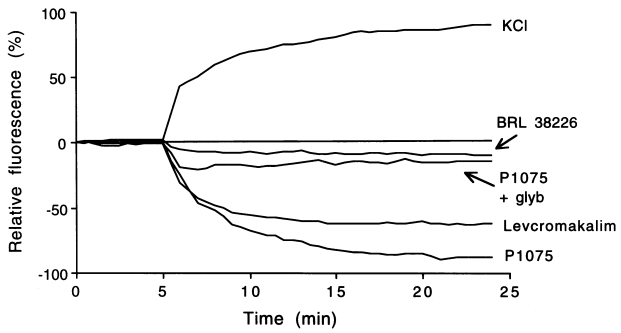

Fig. 2. Effects of KCl and potassium channel openers on OCP cell membrane potential. Potassium channel openers (P1075, 3 μM; levcromakalim, 10 μM; BRL-38226, 10 μM) were added to OCP cells and cell-associated fluorescence measurements were made every min using FLIPR™. Fluorescence intensity was normalized to that produced maximally by 30 mM KCl (100%). Experiments with glyburide (10 μM) were performed on cells preincubated for 15 min prior to potassium channel opener addition. Results show mean data of 12 wells from representative experiments which were performed 2–5 times.

100%. P1075 (3 μM) hyperpolarized OCP cells (monitored as decreased fluorescence) and the response was maximal after 20 min (Fig. 2). The response was concentration-dependent with 10 μM P1075 producing 70–100% of the fluorescence change seen with 30 mM KCl. Preincubation of cells with 10 μM glyburide for 15 min inhibited the P1075-mediated hyperpolarization. Levcromakalim (10 μM) also hyperpolarized OCP cells and its 3*R*,4*S* enantiomer, BRL 38226 was significantly less active (Fig. 2), which indicates that the hyperpolarization response to cromakalim was stereoselective. Responses to the cromakalim enantiomers were also inhibited by glyburide (data not shown). Thus, OCP cells can be hyperpolarized by potassium channel openers in a glyburide-sensitive manner, supporting the view that these cells contain K_{ATP} channels.

4. Discussion

Evidence was obtained indicating the presence of K_{ATP} channels in an epithelial cell line from ovine choroid plexus. Thus, the present study demonstrated the presence of binding sites for the K_{ATP} channel opener [^{3}H]P1075, patch clamp data showing a glyburide-sensitive outward potassium current and glyburide-sensitive, stereoselective, hyperpolarization response to potassium channel openers.

Quast et al. first described the use of the potent potassium channel opener P1075 to label sites on aortic smooth muscle cells and showed that their characteristics were similar to those which mediated smooth muscle relaxation (Bray and Quast, 1992; Quast et al., 1993). These data implied that [^{3}H]P1075 binding sites were associated with K_{ATP} channels. Specific, saturable, [^{3}H]P1075 binding was observed on OCP and the B_{max} value was approximately twice that for rat aortic smooth muscle cells. The K_d of [^{3}H]P1075 for OCP cells was 26 nM which was higher than K_d values of 4 and 6 nM which were reported for rat aortic smooth muscle (Dickinson et al., 1993; Bray and Quast, 1992) but similar to a K_d of 32 nM determined for canine cardiomyocyte (Dickinson et al., 1993). Based upon the compounds examined, it appears that the pharmacological characteristics of OCP and cardiac [^{3}H]P1075 binding sites were similar. Thus, the pinacidil analogs exhibited a rank order of potency: P1075 > ((4-[[9cyanoimino)[(1,2,2-trimethylpropyl)amino]-methyl]amino]benzonitrile(BMS-182264) > pinacidil; levcromakalim was significantly more potent than its 3*R*,4*S* enantiomer BRL 38226; and the potassium channel openers nicorandil, and diazoxide were relatively poor inhibitors of binding.

The presence of K_{ATP} channels or processes mediated via this channel are generally characterized by inhibition with the K_{ATP} channel blocker glyburide. Recent evidence suggests that K_{ATP} channels are composed of an inward rectifying potassium channel in association with a sulfonylurea receptor which confers glyburide sensitivity on IK_{ATP} (Inagaki et al., 1995). Recent literature indicate that CFTR and ROMK2 can also form an ATP-regulated K^+ channel which is sensitive to glyburide (McNicholas et al., 1996). However, to date, CFTR has not been detected in choroid plexus cells (Kibble et al., 1996). Glyburide inhibited all potassium channel opener-mediated events in OCP cells including [^{3}H]P1075 binding, P1075-mediated outward current and cellular hyperpolarization. The potency of glyburide as an inhibitor of [^{3}H]P1075 binding was slightly lower than Ki values of 490 nM for rat aortic smooth muscle cells (Dickinson et al., 1993) and 650 nM for canine cardiomyocytes (unpublished data). However, the potency of glyburide as an inhibitor of K_{ATP} channels can be markedly decreased by the presence of ADP (Venkatesh et al., 1991). Inhibition of P1075-mediated effects by glyburide is unlikely to be competitive. Thus, previous studies showed that glyburide increased the rate of [^{3}H]P1075 dissociation from rat aortic rings in a manner similar to that of an allosteric regulator (Bray and Quast, 1992). We obtained similar results with OCP cells. Thus, a combination of glyburide and excess P1075 dissociated [^{3}H]P1075 from OCP cells more significantly than P1075 alone (data not shown). These data are consistent with an allosteric interaction of glyburide and potassium channel opener binding sites on OCP cells.

Examination of OCP cells by patch clamp studies defined a glyburide-sensitive, P1075-activated, outward K^+ current, which also suggested the presence of K_{ATP} channels. However, a clear response to P1075 was obtained in only a fraction of cells indicating substantial cell to cell variation in channel expression. Further evidence for the presence of K_{ATP} channels in OCP cells was provided by membrane potential changes in confluent cell monolayers. Since K_{ATP} channels are associated with relatively large conductance changes, it is likely that activation of a small number of channels could result in significant K^+ flux and hyperpolarization. Indeed, membrane potential sensitive dyes have been successfully used to monitor P1075-mediated hyperpolarization in A10 smooth muscle cells (Holevinsky et al., 1994) and we observed similar findings with OCP cells. Thus, P1075 hyperpolarized OCP cells in a glyburide-sensitive and dose-dependent manner. Moreover, the hyperpolarization response was stereoselective for the 3*S*,4*R* enantiomer of cromakalim. These results were similar to those observed for the [^{3}H]P1075 binding sites and are characteristic of an interaction with K_{ATP} channels. Taken together with the patch clamp data, we conclude that K_{ATP} channels are present on choroid plexus cells. The physiological significance of choroid plexus K_{ATP} channels remains to be established. However, K_{ATP} channels clearly exist on other epithelial cells such as frog skin principal cells where they are involved in K^+ recycling across basolateral membranes (Urbach et al., 1996). In kidney epithelial cells, they may also play important roles in determining K^+ conductive pathways along the nephron (Welling, 1995).

In summary, we have characterized the [^{3}H]P1075 binding sites on choroid plexus cells. The presence of K_{ATP} channels was also suggested by patch clamp and membrane potential measurements. It is likely that [^{3}H]P1075 binds to a component of the K_{ATP} channel complex in epithelial cells.

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