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Phosphoinositol metabolism affects AMP kinase-dependent K-ATP currents in rat substantia nigra dopamine neurons

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Abstract

We reported recently that ligand-gated ATP-sensitive K⁺ (K-ATP) current is potentiated by AMP-activated protein kinase (AMPK) in rat substantia nigra compacta (SNC) dopamine neurons. Because phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) regulates K-ATP current, we explored the hypothesis that changes in PI(4,5)P₂ modify the ability of AMPK to augment K-ATP current. To influence PI(4,5)P₂ levels, we superfused brain slices with phospholipase C (PLC) activators and inhibitors while recording whole-cell currents in SNC dopamine neurons. Diazoxide, superfused for 5 min every 20 min, evoked K-ATP currents that, on average, increased from 38 pA at first application to 122 pA at the fourth application, a 220% increase. This enhancement of diazoxide-induced current was AMPK dependent because K-ATP current remained at baseline when slices were superfused with either the AMPK inhibitor dorsomorphin or the upstream kinase inhibitor STO-609. The PLC inhibitor U73122 significantly increased diazoxide current over control values, and this increase was blocked by dorsomorphin. Enhancement of diazoxide-induced current was also completely prevented by the PLC activator m-3M3FBS. Agonists at 5-HT_{2C} and group I metabotropic glutamate receptors, both of which activate PLC, also prevented augmentation of diazoxide-induced current. Finally, inhibition of spike discharges by diazoxide was significantly antagonized by m-3M3FBS. These results suggest that PLC activity significantly influences the inhibitory effect of K-ATP channels by altering PI(4,5)P₂ content. Results also suggest that modification of K-ATP current by PLC requires AMPK activity.

Keywords

ATP-sensitive potassium channel; dopamine neuron; diazoxide; AMP kinase; patch clamp recording; brain slice

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1. Introduction

The ATP-sensitive K⁺ (K-ATP) channel is an octomeric complex composed of four sulfonylurea receptor subunits and four inwardly rectifying K⁺ channel subunits (Aguilar-Bryan and Bryan, 1999). These channels respond to the metabolic state of the cell in that they close when ATP levels are high and open when ATP is depleted. They are probably best characterized in pancreatic beta-cells where closure of the channel by ATP causes membrane depolarization and calcium influx and thereby triggering insulin release (Ashcroft, 2007). Because the K-ATP channel is regulated by changes in high energy phosphates, it serves as an important cellular metabolic sensor (Miki and Seino, 2005).

5'-Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric enzyme complex that also responds to changes in cellular energy levels (Hardie, 2014). When ATP levels fall and AMP rises, AMPK institutes a number of cellular processes that lead to reductions in energy expenditure and increases in ATP production (Hallows, 2005). Although AMP is necessary, AMPK activation requires phosphorylation by the upstream kinase LBK1 or CaMKK β (Carling et al., 2008). AMPK and K-ATP channels are functionally related in the pancreas, where AMPK activation has been shown to facilitate translocation of K-ATP channels to the cell surface (Chen et al., 2013; Park et al., 2013b). AMPK has also been shown to augment sulfonylurea-sensitive current in cardiac myocytes (Yoshida et al., 2012). Although both AMPK and K-ATP channels are widely expressed in brain (Turnley et al., 1999; Dunn-Meynell et al., 1998), a functional interaction between these important cellular constituents needs further characterization.

Dopamine neurons in the substantia nigra zona compacta (SNC) project primarily to the striatum and are important in maintaining normal movement as well as mediating aspects of behavioral reinforcement and motivation (Schultz et al., 1993; Young and Penney, 1984). It is important to understand how the excitability of dopamine neurons is regulated by synaptic inputs and intrinsic membrane properties if we are to devise pharmacological interventions to manage human conditions that are influenced by dopamine. We have recently reported that K-ATP channel function is augmented by AMPK activation in SNC dopamine neurons (Shen et al., 2016; Wu et al., 2017). This may be important because K-ATP channel activation can hyperpolarize these cells and limit dopamine release when ATP is depleted such as in conditions of metabolic stress. Also, K-ATP current has been reported to facilitate burst firing in a subset of SNC neurons (Schiemann et al., 2012; Dragicevic et al., 2014), and burst firing is known to greatly facilitate dopamine release in target nuclei (Gonon and Buda, 1985; Manley et al., 1992). Although it is not clear how AMPK facilitates K-ATP channel function in SNC neurons, it is well known that the opening probability of K-ATP channels is strongly regulated by membrane levels of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂). Because it diminishes the potency of ATP to block K-ATP channels, PI(4,5)P₂ is essential for K-ATP channels to open under physiological conditions (Krauter et al., 2001; Gamper and Rohacs, 2012). Therefore, we were interested in characterizing the effect of altering PI(4,5)P₂ levels on K-ATP channel function in the presence and absence of AMPK activity.

In the present study, we used patch pipettes to record from rat SNC dopamine neurons in the whole-cell configuration. We used activators and inhibitors of phospholipase C (PLC) in

order to study changes in PI(4,5)P₂ on currents evoked by the K-ATP channel opener diazoxide. Furthermore, we used specific blockers of AMPK activation in order to characterize the dependence of diazoxide-induced currents on AMPK. Results suggest that alterations in PI(4,5)P₂ have marked effects on K-ATP channel function in SNC dopamine neurons. Moreover, the modification of K-ATP currents is dependent upon AMPK activity.

2. Results

2.1 Augmentation of K-ATP current is AMPK-dependent

Recent work from our lab showed that currents evoked in SNC dopamine neurons by K-ATP channel openers gradually increase in amplitude during patch clamp recordings in the whole-cell configuration (Shen et al., 2016). In the present study slices were superfused with the K-ATP channel opener diazoxide (200 μ M) for 5 min every 20 min in order to further investigate changes in diazoxide-induced current recorded over time. As shown in Fig. 1A, currents evoked by diazoxide double or triple in amplitude over the duration of whole-cell recording. Our previous study showed that progressive augmentation of current correlated with the duration of whole-cell recording rather than repetitive exposure to diazoxide (Shen et al., 2016). Moreover K-ATP currents do not develop spontaneously during whole-cell recordings, but rather recording in the whole-cell configuration increases the capacity for evoking K-ATP current over time (Wu et al., 2017; Shen et al., 2016). Although the mechanism by which whole-cell recording augments K-ATP-evoked current is not clear, our data suggests that augmentation of diazoxide-induced current is dependent upon AMPK activation. Figure 1B shows that bath application of the AMPK blocker dorsomorphin (20 μ M) completely prevents augmentation of diazoxide-induced current. We showed previously that dorsomorphin, which is also known as compound C, prevents activation of AMPK by inhibiting Thr-172 phosphorylation in rat midbrain slices (Shen et al., 2014). Figure 1C shows that augmentation of diazoxide-induced current was prevented by either bath application of dorsomorphin (20 μ M; $n = 5$) or when dorsomorphin was contained in the pipette internal solution (5 μ M; $n = 10$). There was a significant main effect on diazoxide-induced current when pipettes contained dorsomorphin ($F_{(1,17.92)} = 19.67$, $P = 0.000322$). Under control conditions, the fourth application of diazoxide (60 min) evoked 122 ± 24 pA ($n = 8$) of outward current, which was significantly greater than the 39 ± 13 pA ($n = 10$) of outward current when pipettes contained 5 μ M dorsomorphin ($P < 0.001$, Sidak paired comparison). In addition, we tested the effect of STO-609, which prevents AMPK activation by blocking the upstream kinase CaMKK β (Hawley et al., 2005). As shown in Fig. 1D, there was a significant main effect of STO-609 (20 μ M) on diazoxide-induced current ($F_{(1,18.59)} = 14.20$, $P = 0.001344$). At the fourth application (60 min), diazoxide evoked only 49 ± 6 pA ($n = 9$) of outward current compared to 122 ± 24 pA of outward current under control conditions ($P < 0.001$, Sidak pairwise comparison). These results further support the conclusion that AMPK activation mediates the progressive augmentation of diazoxide-induced current during whole-cell recordings.

It should be noted that the above experiments were conducted in superfusate that contained 11 mM glucose. Although relatively high, previous studies suggested that this concentration of glucose can prevent ischemia and preserve synaptic functions in slice preparations

(Whittingham et al., 1984; Schurr et al., 1989). Consequently, superfusate containing 10–11 mM glucose has been standard practice in brain slice preparations for decades (Alger et al., 1984). Nevertheless, it is known that glucose levels can influence levels of ATP and other nucleotides that can alter AMPK as well as K-ATP channel function. Therefore, we repeated experiments using superfusate containing 3 mM glucose, which is within the physiological range for CSF (Adams and Victor, 1989; Silver and Erecinska, 1994). Slices were superfused with 3 mM glucose at least 2 hrs before starting recordings. Figure 1E shows that the amplitudes of diazoxide-induced currents were augmented during whole-cell recordings. When comparing control diazoxide-induced currents in Fig. 1E to those in Fig. 1C, mixed model analysis showed there was no significant effect of recording in 3 mM versus 11 mM glucose ($F_{(1,16.16)} = 0.59$, $P = 0.453$). Figure 1E also shows that dorsomorphin (20 μ M in the bath) completely prevented the augmentation of diazoxide-induced currents recorded in 3 mM glucose superfusate. Statistical analysis shows there was a significant main effect on diazoxide-induced current when recording with dorsomorphin ($F_{(1,14.02)} = 75.46$, $P = 5.23 \times 10^{-7}$). Under control conditions, the fourth application of diazoxide (60 min) evoked 105 ± 37 pA ($n = 8$) of outward current, which was significantly greater than the 6 ± 1 pA ($n = 6$) of outward current when superfusate contained dorsomorphin ($P < 0.001$, Sidak paired comparison). These results suggest that the dorsomorphin-sensitive augmentation of diazoxide-induced currents occurs in superfusate containing 3 mM as well as 11 mM glucose. Subsequent experiments were conducted in superfusate that contained 11 mM glucose.

2.2 PLC inhibition further augments diazoxide-induced current

The potency of ATP to block K-ATP channels is dramatically increased when membrane lipids contain low levels of the phospholipid PI(4,5)P₂ (Baukrowitz and Fakler, 2000; Shyng et al., 2000). If PLC is tonically active in SNC dopamine neurons, we reasoned that inhibition of PLC might further augment diazoxide-induced currents. Figure 2A shows that superfusing the slice with the PLC inhibitor U73122 (Bleasdale et al., 1990) enhanced currents evoked by diazoxide (200 μ M). Superfusion with U73122 (5 μ M) was begun 5 min after the first application of diazoxide. Moreover, diazoxide-induced currents were reduced when U73122 was superfused with dorsomorphin, as shown in Fig. 2B. Figure 2C is a summary graph showing that there was a significant main effect of treatment with U73122 on diazoxide-induced currents ($F_{(1,27.48)} = 8.35$, $P = 0.001344$). During the second application of diazoxide (20 min), diazoxide-induced current was significantly larger in U73122 (152 ± 15 pA, $n = 19$) compared to the control value of 84 ± 15 pA, $n = 10$ ($P < 0.001$, Sidak pairwise comparison). In contrast, there was virtually no enhancement of diazoxide-induced currents when U73122 was superfused with dorsomorphin ($n = 5$). These data suggest that PLC is tonically active in the SNC, and inhibition of PLC potentiates K-ATP currents by elevating membrane levels of PI(4,5)P₂. Moreover, the ability of dorsomorphin to block the effect of U73122 suggests that AMPK activity is needed to observe the effect of PLC inhibition.

2.3 PLC activation blocks enhancement of diazoxide-induced currents

We next tested the hypothesis that an activator of PLC would prevent the enhancement of diazoxide-induced currents seen during whole-cell recordings. Superfusion with the PLC

activator m-3M3FBS (Bae et al., 2003) was begun 5 min after the first application of diazoxide. The current trace in Fig. 3A shows that m-3M3FBS (25 μ M) prevented the augmentation of diazoxide-evoked current. As seen in the summary graph in Fig. 3B, there was a significant main effect of m-3M3FBS on diazoxide-induced currents ($F_{(1,18.48)} = 38.38$, $P = 0.000006$). During the fourth application of diazoxide (60 min), diazoxide-induced current was significantly smaller in m-3M3FBS (20 ± 4 pA, $n = 8$) compared to the control value ($P < 0.001$, Sidak pairwise comparison). These results are consistent with those of others who showed that K-ATP channels require sufficient membrane levels of PI(4,5)P₂ to sustain open probability (Gamper and Rohacs, 2012).

2.4 Effects of 5-HT_{2C} and mGluR agonists

The 5-HT_{2C} receptor is known to activate PLC (Wolf and Schutz, 1997). Moreover, the SNC receives robust innervation from serotonergic nuclei (Fibiger and Miller, 1977). We therefore explored the effect of m-CPP, a 5-HT_{2C} agonist (Callahan and Cunningham, 1994), on diazoxide-induced currents. The current trace in Fig. 4A shows that superfusion with m-CPP (30 μ M) prevents the augmentation of diazoxide-induced currents during whole-cell recording. As seen in the summary graph in Fig. 4B, there was a significant main effect of m-CPP on diazoxide-induced currents ($F_{(1,14.02)} = 5.41$, $P = 0.0355$). During the fourth application of diazoxide (60 min), diazoxide-induced current was significantly smaller in m-CPP (64 ± 19 pA, $n = 6$) compared to the control value ($P < 0.05$, Sidak pairwise comparison).

We also examined the effect of DHPG, which is a group I metabotropic glutamate receptor (mGluR) agonist that is known to activate PLC (Conn and Pin, 1997). Superfusion with DHPG (3 μ M) inhibits the augmentation of diazoxide-induced current as shown in Fig. 5A. Figure 5A also shows that DHPG evokes an inward current, which has been shown to be caused by a mixed cationic current in SNC neurons (Shen and Johnson, 1997; Bengtson et al., 2004). As seen in the summary graph in Fig. 5B, there was a significant main effect of DHPG on diazoxide-induced currents ($F_{(1,17.61)} = 7.95$, $P = 0.011502$). During the fourth application of diazoxide (60 min), diazoxide-induced current was significantly smaller in DHPG (66 ± 14 pA, $n = 9$) compared to the control value ($P < 0.01$, Sidak pairwise comparison). These results suggest that activation of endogenous neurotransmitter receptors can inhibit K-ATP channel function, which is consistent with effects mediated by PLC activation.

2.5 Effect of PLC activation on neuronal excitability

To illustrate the interactive effect of PLC and K-ATP channels on neuronal excitability, we used depolarizing current pulses to evoke action potentials under current clamp. As shown in Fig. 6A, superfusing the slice with diazoxide (200 μ M) for 5 min caused a marked reduction in spike generation. Moreover, this effect of diazoxide was severely reduced by superfusate that contained the PLC activator m-3M3FBS (25 μ M). Diazoxide-induced current was measured after a 20 min superfusion with m-3M3FBS and compared to current without m-3M3FBS in a separate population of cells. Under control conditions, diazoxide reduced spike frequency to 2.6 ± 1.2 Hz ($n = 6$), which was significantly smaller than the 17.2 ± 3.5 Hz ($n = 6$) recorded before diazoxide ($P = 0.003$, paired t test). But in the presence of

m-3M3FBS, firing rate in diazoxide was 11.9 ± 2.0 Hz ($n = 8$) compared to the rate of 15.5 ± 1.6 Hz ($n = 8$) before diazoxide. Fifteen min after diazoxide was washed out, firing rate recovered fully in the presence (14.6 ± 1.7 Hz, $n = 8$) and absence (15.5 ± 3.2 Hz, $n = 6$) of m-3M3FBS. Figure 6B shows these data normalized in terms of percent reduction in firing rate caused by diazoxide. Diazoxide caused an $88.7 \pm 5.3\%$ reduction in firing rate under control conditions ($n = 6$), which was significantly greater than the $24.6 \pm 8.2\%$ reduction in firing rate recorded in m-3M3FBS ($n = 8$; $t = 6.057$, $P = 0.000057$, t test). These data suggest that PLC activity and resulting alterations in membrane levels of PI(4,5)P₂ greatly influence the inhibitory effect of K-ATP channel activation on neuronal excitability.

3. Discussion

The present study extends our understanding of how K-ATP currents are regulated in SNC dopamine neurons. We showed previously that ligand-gated K-ATP currents progressively increase in amplitude during whole-cell patch-clamp recordings (Shen et al., 2016). We also showed that K-ATP current does not develop spontaneously during whole-cell recording, but rather the capacity for a cell to generate K-ATP current increases during the recording (Wu et al., 2017). Involvement with AMPK was suggested by the findings that K-ATP current was further augmented by the AMPK activator A769662 and inhibited by dorsomorphin (compound C), which blocks AMPK activation by preventing its phosphorylation (Shen et al., 2016). Involvement with AMPK was further supported by results of the present study showing that augmentation of diazoxide-induced currents is prevented by STO-609, which inhibits activation of the upstream kinase CaMKK β , or when dorsomorphin is present in the recording pipette. Finally, our previous studies showed that augmentation of ligand-gated K-ATP current during whole-cell recording is not a feature of non-dopamine neurons in the subthalamic nucleus even though AMPK activation augments calcium-dependent generation of K-ATP current in these neurons (Shen et al., 2014). At this stage of our research, two questions remain: 1) how does whole-cell recording activate AMPK, and 2) how does AMPK augment ligand-gated K-ATP current? The present study was done in an effort to explore the second question, and specifically, to explore the possibility that AMPK augments K-ATP current by altering phosphoinositol metabolism.

PI(4,5)P₂ is well known to affect neuronal excitability by virtue of its ability to enhance the function of a variety of ion channels including inwardly rectifying K⁺ channels, hyperpolarization-activated cyclic nucleotide-gated channels, and transient receptor potential channels (Ryan and Sanders, 1993; Ying et al., 2011; Hille et al., 2015). In the case of K-ATP channels, PI(4,5)P₂ influences channel function by significantly reducing the potency of ATP to block K-ATP channels (Baukrowitz et al., 1998; Shyng et al., 2000). Because PLC metabolizes PI(4,5)P₂, we used PLC activators and inhibitors to investigate how changes in PI(4,5)P₂ levels affect K-ATP channel function in SNC dopamine neurons. Our studies showed that the PLC inhibitor U73122 significantly increased diazoxide-induced current; this suggests that PLC is tonically active, and K-ATP currents are enhanced by reducing the metabolic degradation of PI(4,5)P₂. Moreover, the effect of U73122 could be blocked by the AMPK blocking agent dorsomorphin, which suggests that AMPK activity is required for PLC inhibition to enhance K-ATP channel function. Involvement of PI(4,5)P₂ is also supported by our finding that the PLC activator m-3M3FBS blocked the augmentation of

diazoxide-induced currents during whole-cell recording. The 5HT_{2C} and mGluR agonists m-CPP and DHPG, which also activate PLC, also inhibited diazoxide-induced current. Our results suggest that membrane content of PI(4,5)P₂, which is influenced by PLC activity, has significant effect on K-ATP channel function in SNC dopamine neurons. To our knowledge, ours is the first report of altered excitability of SNC dopamine neurons by PI(4,5)P₂-dependent changes in K-ATP channel function. Although the influence of PI(4,5)P₂ on K-ATP channel function is well established (Gamper and Rohacs, 2012), our finding that AMPK is required for PI(4,5)P₂ to exert an influence is novel. A schematic illustrating our findings is shown in Fig. 7.

A major question to be addressed is whether or not AMPK augments K-ATP channel function by altering phosphoinositol metabolism. Phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) is a phosphoinositol metabolite that facilitates the trafficking of protein complexes to the cell surface, such as the GLUT4 glucose transporter (Billcliff and Lowe, 2014) and the AMPA receptor (Seebold et al., 2012). Moreover, AMPK has been shown to stimulate phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), which is a kinase that generates PI(3,5)P₂ (Lang and Föller, 2014; Liu et al., 2015). Although it is not known if augmentation of diazoxide-induced currents is due to AMPK-dependent translocation of K-ATP channels to the cell surface, it is possible that AMPK-dependent increases in PI(3,5)P₂ levels could mediate such an effect. AMPK also has been reported to inhibit phosphatidylinositol 3,4,5 triphosphate 3-phosphatase (PTEN), which facilitates the formation of PI(3,4,5)P₃ and subsequently PI(3,5)P₂ (Park et al., 2013a). These investigators also showed that inhibition of PTEN facilitated the trafficking of K-ATP channels to the cell surface in pancreatic beta-cells. Thus, it appears that there are several ways in which AMPK activation might facilitate K-ATP channel function via alterations in phosphoinositol metabolism. One should note that a direct effect of AMPK on PI(4,5)P₂ levels has not, to our knowledge, been established. In fact, inhibition of PTEN by AMPK would be expected to reduce the conversion of PI(3,4,5)P₃ to PI(4,5)P₂. It is possible that AMPK activity is necessary but not sufficient for K-ATP channel function. Nevertheless, our studies clearly show that alterations in PI(4,5)P₂ by PLC activity has a dramatic effect on K-ATP function in SNC dopamine neurons, and this requires AMPK activity.

Our results also suggest a potentially significant interaction between neurotransmitter receptors and K-ATP channel function. Both group I mGluR and 5-HT₂ receptor agonists activate PLC and are widely expressed in the basal ganglia (de Deurwaerdère et al., 2013; Rouse et al., 2000). Our studies show that the group I mGluR agonist DHPG and the 5-HT_{2C} agonist m-CPP prevented the augmentation of diazoxide-evoked currents, most likely via their ability to activate PLC. The potential effect of PLC activation on neuronal excitability was illustrated by our report that the PLC activator m-3M3FBS dramatically reduced the inhibitory effect of diazoxide on evoked firing rate. Because both group I mGluR and 5-HT₂ receptor agonists have excitatory actions on midbrain dopamine neurons (Guatteo et al., 1999; Pessia et al., 1994), it is possible that excitatory effects of receptor stimulation could be reinforced by their abilities to antagonize tonic K-ATP channel activity, which might be especially relevant when these channels are opened when ATP levels are reduced during times of metabolic stress. Although short-term opening of K-ATP channels may be neuroprotective (Tai and Truong, 2002; Abele and Miller, 1990), prolonged opening of K-

ATP channels have been shown to promote cell death and has been suggested as a possible risk factor in dopamine neurodegeneration in Parkinson's disease (Dragicevic et al., 2014; Toulorge et al., 2010). Thus, it is possible that PLC activation by endogenous neurotransmitters could provide some neuroprotection by diminishing the influence of K-ATP channel opening. Clearly, it would be of interest to further explore the consequences and potential benefits of modulating K-ATP channel function by altering phosphoinositol metabolism.

It should be noted that metabolic stressors such as hypoglycemia and hypoxia are well known to activate K-ATP channels in SNC dopamine neurons (Guatteo et al., 1998; Marinelli et al., 2000). Although metabolic stress also activates AMPK (Hardie, 2014), our previous work showed that AMPK activation is not sufficient by itself to trigger K-ATP current, but rather it increases the capacity to generate K-ATP current (Shen et al., 2016; Wu et al., 2017). Although not intended to be a focus of the present study, it is interesting to speculate on how recording in the whole-cell configuration can activate AMPK in dopamine neurons. Increases in intracellular calcium can activate AMPK via stimulation of CaMKK β (Carling et al., 2008), and it is possible that whole-cell recordings disturb calcium homeostasis. AMPK could also be activated by accumulation of AMP caused by metabolism of ATP that is contained in pipette solutions. Although the mechanism for AMPK activation is as yet unclear, we contend that AMPK activation during whole-cell recordings likely mimics a process that may occur naturally during times of metabolic stress.

In conclusion, our studies suggest that alterations in membrane levels of PI(4,5)P₂ can dramatically affect dopamine neuronal excitability due to changes in K-ATP channel function. Results also suggest that the inhibitory effect of K-ATP channels can be overcome by 5HT_{2C} and mGluR agonists by virtue of their ability to activate PLC and reduce PI(4,5)P₂ content. Further studies are needed to explore the possibility that specific phosphoinositol metabolites underlie the ability of AMPK to augment K-ATP channel function in dopamine neurons. Because regulation of K-ATP channels has been shown to differ amongst different types of central neuron (Shen et al., 2014; Karunasinghe et al., 2017), it cannot be assumed that results of our study will be applicable to all neuronal types. Future studies will be needed to investigate the interactions between AMPK and K-ATP channels in a variety of central neurons.

4. Experimental Procedures

4.1 Animals and preparation of brain slices

A total of 75 adult male rats were used in this study. Animal care and euthanasia procedures were performed as approved by the Institutional Animal Care and Use Committee at the VA Portland Health Care System. Male Sprague-Dawley rats (100–180 g, 3–7 weeks old) were obtained from Harlan (Indianapolis, IN). Every care was made to reduce stress and the number of rats used. Rats were euthanized by severing major thoracic vessels under isoflurane anesthesia and the brain was quickly removed. Horizontal midbrain slices (300 μ m thick) were cut on a vibratome in an ice-cold solution composed of (in mM): sucrose (196); KCl (2.5); MgCl₂ (3.5); CaCl₂ (0.5); NaH₂PO₄ (1.2); glucose (20); and NaHCO₃ (26). Slices were placed in an oxygenated solution of the same composition used for whole-

cell recording and allowed to equilibrate at room temperature for one hour before being placed in the recording chamber. Recording solution had the following composition (in mM): NaCl (126); KCl (2.5); CaCl₂ (2.4); MgCl₂ (1.2); NaH₂PO₄ (1.2); NaHCO₃ (19); glucose (11), gassed with 95% O₂ and 5% CO₂ (pH 7.4) at 36°C. In some experiments superfusate contained 3 mM rather than 11 mM glucose, with osmolality balanced with additional NaCl. Slices were submerged and superfused continuously at a rate of 2–3 ml/min.

4.2 Whole-cell recordings and identification of SNC neurons

Using a dissection microscope, the SNC was identified as gray matter located just rostral to the medial terminal nucleus of the accessory optic tract. Borosilicate micropipettes were filled with internal solution of the following composition (in mM): potassium gluconate (138); MgCl₂ (2); CaCl₂ (1); EGTA (11); HEPES (10); ATP (1.5); GTP (0.3), at pH 7.3. Beginning pipette resistance ranged from 2–5 MΩ and recordings were discarded if resistance rose to 30 MΩ. Potentials were clamped at –60 mV during voltage-clamp recordings. Membrane currents and voltages were recorded with an Axopatch-1D amplifier (Molecular Devices, Foster City, CA, USA) using pClamp 10 and Axoscope software (Molecular Devices) run on a personal computer. Action potentials were sampled at 10 kHz with a 2 kHz low-pass filter. Long-term voltage-clamp recordings were sampled at a rate of 500 Hz. Dopamine neurons were identified using well-established electrophysiological characteristics such as spontaneous firing rates of 1–5 Hz, relatively broad action currents, and presence of large hyperpolarization-activated inward currents (H-current) (Grace and Bunney, 1983; Lacey and North, 1988).

4.3 Drugs and chemicals

Drugs and chemicals were dissolved in water or DMSO as stock solutions before being added to superfusate at 1:1000 dilutions. Control studies showed no effect of this DMSO concentration on membrane conductance or firing rate. Superfusate containing a chemical reached the recording chamber within 1 min after passing through a heat exchanger. The K-ATP channel opener diazoxide was superfused for 5 min every 20 min; net diazoxide-induced currents were measured as the difference between peak current and current immediately before application of diazoxide. Test agents (dorsomorphin, STO-609, U73122, m-3M3FBS, m-CPP, and DHPG) were added to the superfusate 5 min after the first application of diazoxide. The first application of diazoxide was begun about 10 min after starting whole-cell recording. In some experiments dorsomorphin was added to the pipette internal solution and allowed to diffuse passively into the cell. Diazoxide was obtained from Sigma-Aldrich (St. Louis, MO, USA), m-CPP (meta-chlorophenylpiperazine) was from Tocris Cookson (Bristol, UK), dorsomorphin and STO-609 were from Cayman Chemical (Ann Arbor, MI, USA), U73122 and m-3M3FBS were from R&D Systems (Minneapolis, MN, USA), and DHPG ((S)-3,5-dihydroxyphenylglycine) was from Ascent Scientific (Cambridge, MA, USA).

4.4 Data analysis

Numerical data are expressed as means and standard errors throughout the text. Data obtained at multiple time points were analyzed for statistical significance using a linear

mixed model run on a personal computer with IBM SPSS Statistics version 22 (IBM North America, New York, NY, USA). Normality of linear mixed model datasets were confirmed using Studentized residual plots of logarithmically transformed data. Other data sets were tested with paired or unpaired *t* tests where appropriate. All data sets were tested for normality before analysis. Significance was accepted with $P < 0.05$.

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Abbreviations:

AMPK	AMP-activated protein kinase
K-ATP channel	ATP-sensitive K ⁺ channel
mGluR	metabotropic glutamate receptor
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
SNC	Substantia nigra zona compacta

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Highlights

- AMPK activation potentiates K-ATP current in SNC dopamine neurons
- PLC inhibition further potentiates K-ATP currents and is AMPK dependent
- PLC activation by 5HT and mGluR agonists block potentiation of K-ATP currents
- Alteration in neuronal excitability by PLC requires AMPK activation

Fig. 1.

Augmentation of diazoxide-induced currents is AMPK-dependent. A) Current trace shows that repetitive bath applications of diazoxide (200 μ M) evoke progressively larger outward currents. In this and in subsequent experiments slices were superfused with diazoxide for 5 min every 20 min. B) Augmentation of diazoxide-induced current is prevented by superfusing the slice with dorsomorphin (20 μ M). C) Summary graph showing that augmentation of diazoxide-induced currents is prevented by dorsomorphin that was either added to the superfusate (20 μ M) or to pipette solutions (5 μ M). Time at “zero” min represents the time of peak current recorded during the first application of diazoxide. D) Augmentation of diazoxide-induced current was blocked by bath application of STO-609 (20 μ M). E) Summary data of diazoxide-induced currents recorded in superfusate containing 3 mM rather than the usual 11 mM glucose. Augmentation of diazoxide-induced currents in 3 mM glucose was comparable to that recorded in 11 mM glucose. The graph also shows

that dorsomorphin blocked augmentation of diazoxide- induced currents in 3 mM glucose superfusate. Sidak pairwise comparison tests: **, $P < 0.01$; ***, $P < 0.001$.

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Fig. 2.

The PLC inhibitor U73122 potentiates diazoxide-induced currents. A) Current trace showing large diazoxide-induced currents recorded in superfusate that contained U72133 (5 μ M). Note that superfusion with U73122 began 5 min after the first diazoxide application. B) Current trace showing that diazoxide-induced currents are progressively smaller when recorded with both U73122 and dorsomorphin (20 μ M) in the superfusate. C) Summary graph showing that U73122 significantly increased the amplitudes of diazoxide-induced currents. The graph also shows that dorsomorphin blocked the ability of U73122 to augment diazoxide-induced currents. Sidak pairwise comparison tests for U73122 vs control: *, $P < 0.05$; ***, $P < 0.001$.

Fig. 3.

The PLC activator m-3M3FBS (25 μ M) prevents augmentation of diazoxide-induced currents. A) Typical current trace showing the effect of bath application of m-3M3FBS on diazoxide-induced currents. B) Summary graph showing that m-3M3FBS prevents augmentation of diazoxide-induced currents. Sidak pairwise comparison tests: **, $P < 0.01$; ***, $P < 0.001$.

Fig. 4. The 5-HT_{2C} agonist m-CPP (30 μ M) prevents augmentation of diazoxide-induced current. A) Typical current trace of diazoxide-induced currents recorded in superfusate containing m-CPP. B) Summary graph showing that m-CPP prevents augmentation of diazoxide-induced currents. Sidak pairwise comparison tests: *, $P < 0.05$.

Fig. 5.

The mGluR agonist DHPG (3 μ M) prevents augmentation of diazoxide-induced current. A) Typical current trace of diazoxide-induced currents recorded in superfusate containing DHPG. Note that DHPG caused a small inward current when first applied, which is consistent with activation of a mixed cation conductance. B) Summary graph showing that DHPG prevents augmentation of diazoxide-induced currents. Sidak pairwise comparison tests: *, $P < 0.05$; **, $P < 0.01$.

Fig. 6.

PLC activation blunts the inhibitory effect of diazoxide on spike discharge. A) Voltage recordings of action potentials evoked in the absence and presence of m-3M3FBS (25 μ M). Spikes were evoked by depolarizing current pulses before, during and after 5 min bath applications of diazoxide (200 μ M). Diazoxide-induced current was measured after a 20 min superfusion with m-3M3FBS and compared to current without m-3M3FBS in separate populations of cells. B) Summary graph showing the percent reduction in firing rates caused by diazoxide in the presence and absence of m-3M3FBS. Non-paired *t* test: ***, $P < 0.001$.

Fig 7.

Schematic summarizing effects of PLC and AMPK on K-ATP currents. PLC activation, which depletes PI(4,5)P₂ and reduces K-ATP current, can be activated by 5HT_{2C} and mGluR1 receptor stimulation. PLC inhibition, which reduces PI(4,5)P₂ metabolism, increases K-ATP current, and this effect requires AMPK activity.